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PAVLOFF
NIKOLAI &
MERSEREAU, P.A.

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**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371**

Attorney's Docket No. 20020011.ORI

International Application No.: PCT/GB00/02873

International Filing Date: 25 July 2000

Priority Date Claimed: 27 July 1999

Title of Invention:

FRAGMENTS OF CELLULAR PRION PROTEIN
AND METHODS USEFUL IN THE DIAGNOSIS
AND TREATMENT OF PRION DISEASES

Applicant(s) for DO/EO/US:

COLLINGE, John
CLARKE, Anthony Russell
WALTHO, Jonathan Peter
JACKSON, Graham Stuart
HOSSZU, Laszlo Luis Pereira

BOX PCT
COMMISSIONER OF PATENTS AND TRADEMARKS
WASHINGTON, D.C. 20231

Dear Sir:

Applicant hereby submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1)).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

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5. X A copy of the International Application as filed (35 U.S.C. 371(c)(2))
- a. is transmitted herewith (required only if not transmitted by the International Bureau.)
- b. X has been transmitted by the Int'l Bureau.
- c. is not required, as the application was filed in the United States Receiving Office (RO/US)
6. A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
- a. are transmitted herewith (required only if not transmitted by the International Bureau).
- b. have been transmitted by the Int'l Bureau.
- c. have not been made; however, the time limit for making such amendments has NOT expired.
- d. have not been made and will not be made.
8. A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. X An unexecuted oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11-16 below concern documents or information included:

11. An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. X A FIRST preliminary amendment.
 A SECOND or SUBSEQUENT preliminary amendment.
14. A substitute specification.
15. A change of power of attorney and/or address letter.

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16. _____ Other items or information:

A hard copy of the Sequence Listing; and
A disk containing the Sequence Listing

17. X The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(S)(1)-(5)):

Search Report has been prepared by the EPO
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paid to USPTO (37 CFR 1.482) \$
No international preliminary examination fee
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fee (37 CFR 1.482) nor international search
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International preliminary examination fee paid
to USPTO (37 CFR 1.482) and all claims
satisfied provisions of PCT Article 33(2)-(4) \$

ENTER APPROPRIATE BASIC FEE AMOUNT: \$1040.00

_____ Surcharge of \$130.00 for furnishing oath later than
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Total number of claims in excess
of 20, times \$18.00 \$ 864.00
Number of independent claims,
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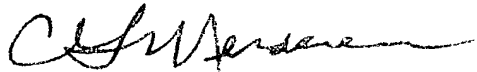
TOTAL FEES ENCLOSED \$2436.00

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- X a. A check in the amount of \$2436.00 to cover the above fees is enclosed.
- X b. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-1265. A duplicate copy of this sheet is enclosed.

Respectfully submitted,

NIKOLAI & MERSEREAU, P.A.



C. G. Mersereau
Attorney for Applicant
Registration No. 26,205
900 Second Avenue South
Suite 820
Minneapolis, MN 55402
Phone: 612-339-7461

MATERIALS AND METHODS USEFUL IN THE DIAGNOSIS AND TREATMENT
OF DISEASES.

REMARKS

In accordance with the above amendment, priority to an earlier co-pending PCT application designating the U.S. is being made within four months of the filing date of the present application and, accordingly, the amendment should be entered.

Respectfully submitted,


NIKOLAI & MERSEREAU, P.A.



C. G. Mersereau
Attorney for Applicant
Registration No. 26205
820 International Centre
900 Second Avenue So.
Minneapolis, MN 55402
(612) 339-7461

CERTIFICATE OF MAILING

I hereby certify that the foregoing Supplemental Preliminary Amendment in application Serial No. 10/031,975, filed on January 24, 2002, of John Collinge et al, entitled "FRAGMENTS OF CELLULAR PRION PROTEIN AND METHODS USEFUL IN THE DIAGNOSIS AND TREATMENT OF PRION DISEASES" is being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to Commissioner of Patents and Trademarks, Washington, D.C. 20231, postage prepaid, on February 12, 2002.



Barbara L. Davis
on behalf of C. G. Mersereau
Attorney for Applicant

Date of Signature: February 12, 2002

1003 10/031975

531 Rec'd PCT/PTC 24 JAN 2002

PATENT APPLICATION

Our Docket No. 20020011.ORI

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re App : John Collinge et al : January 24, 2002
For : FRAGMENTS OF CELLULAR PRION
PROTEIN AND METHODS USEFUL
IN THE DIAGNOSIS AND TREATMENT
OF PRION DISEASES

PRELIMINARY AMENDMENT

Commissioner of Patents and Trademarks

Washington, D.C. 20231

Sir:

Prior to an examination on the merits of the subject application, it is requested that the following amendments be entered:

IN THE CLAIMS

Please cancel original slate containing claims 1-30, without prejudice or disclaimer of any subject matter therein and substitute a new slate containing claims 31-65as follows:

31(New). A peptide selected from the group consisting of all or a fragment or variant of an amino sequence from residue number 176 to 221 of a prion protein cellular form (PrP^c) sequences selected from the group shown in Figure 5.

32(New). A peptide as claimed in claim 31 wherein said amino acid sequence is from residue number 179 to 218 of any one of said PrP^c sequences.

33(New). A peptide selected from the group consisting of

all or a fragment or variant of an amino acid sequence to about ten residues which flank the disulfide bond between Cys 179 and Cys 214 in one of the PrP^C sequences selected from the group consisting of sequences shown in Figure 5.

34(New). A method of making an antibody comprising the steps of:

(a) administering a cellular form (PrP^C) of a prion protein or a peptide selected from the group of peptides consisting of:

- (1) all or a fragment of variant of an amino sequence from residue number 176 to 221 of a prion protein cellular form (PrP^C) sequences selected from the group shown in Figure 5,
- (2) all or a fragment or variant of an amino sequence from residue number 179 to 218 of a prion protein cellular form (PrP^C) sequences selected from the group shown in Figure 5,
- (3) all or a fragment or variant of an amino acid sequence to about ten residues which flank the disulfide bond between Cys 179 and Cys 214 in one of the (PrP^C) sequences selected from the group consisting of sequences shown in Figure 5

to an animal;

- (b) generating an antibody response; and
- (c) collecting the antibody therefrom.

35(New). An antibody obtainable by a method as claimed in

claim 34 which binds preferentially to a cellular form of a prion protein rather than a non-cellular form.

36(New). A method of making a monoclonal antibody comprising the steps of:

(a) administering a cellular form (PrP^c) of a prion protein or a peptide selected from the group of peptides consisting of:

(1) all or a fragment of variant of an amino sequence from residue number 176 to 221 of a prion protein cellular form (PrP^c) sequences selected from the group shown in Figure 5,

(2) all or a fragment or variant of an amino sequence from residue number 179 to 218 of a prion protein cellular form (PrP^c) sequences selected from the group shown in Figure 5,

(3) all or a fragment or variant of an amino acid sequence to about ten residues which flank the disulfide bond between Cys 179 and Cys 214 in one of the (PrP^c) sequences selected from the group consisting of sequences shown in Figure 5

to an animal;

(b) generating an antibody response;

(c) subsequently fusing an antibody producing cell from the animal with a myeloma cell to form a hybridoma; and

(d) obtaining a monoclonal antibody produced by the hybridoma.

37(New). A monoclonal antibody obtainable by a method as claimed in claim 36 which binds preferentially to a cellular form of a prion protein rather than a non-cellular form.

38(New). A method of making a binding agent capable of binding to a cellular form of a prion protein comprising the steps of:

(a) exposing a peptide selected from the group of peptides consisting of:

(1) all or a fragment of variant of an amino sequence from residue number 176 to 221 of a prion protein cellular form (PrP^c) sequences selected from the group shown in Figure 5,

(2) all or a fragment or variant of an amino sequence from residue number 179 to 218 of a prion protein cellular form (PrP^c) sequences selected from the group shown in Figure 5,

(3) all or a fragment or variant of an amino acid sequence to about ten residues which flank the disulfide bond between Cys 179 and Cys 214 in one of the (PrP^c) sequences selected from the group consisting of sequences shown in Figure 5

to a sample whereby any binding agent can bind the peptide; and

(b) collecting a binding agent so bound.

39(New). A method as claimed in claim 38 wherein the binding agent is an antibody.

40(New). A binding agent obtainable by the method of claim 38 which binds preferentially to a cellular form of a prion protein rather than a non-cellular form.

41(New). A method of detecting a cellular form of a prion protein using an antibody as claimed in claim 35 or claim 37 comprising the steps of:

- (a) exposing a sample to the antibody;
- (b) detecting binding of the antibody to the cellular form of a prion protein.

42(New). A method of detecting a cellular form of a prion protein using a binding agent as claimed in either one of claims 39 or 40 comprising the steps of:

- (a) exposing a sample to the binding agent;
- (b) detecting binding of the binding agent to the cellular form of a prion protein.

43(New). A method of detecting a non-cellular form of a prion protein using an antibody as claimed in either claim 35 or claim 37 comprising the steps of:

- (a) exposing a sample to the antibody;
- (b) exposing the sample to an agent which binds the non-cellular form of a prion protein; and
- (c) detecting binding of the antibody to the non-cellular form.

44(New). A method of detecting a non-cellular form of a prion protein using a binding agent as claimed in either claim 39 or claim 40 comprising the steps of:

- (a) exposing a sample to the binding agent;
- (b) exposing the sample to an agent which binds the non-cellular form of a prion protein; and
- (c) detecting binding of the agent to the non-cellular form.

45(New). A method of claim 43 wherein the antibody which has bound the cellular form is separated from the sample prior to step (b).

46(New). A method of claim 44 wherein the binding agent which has bound the cellular form is separated from the sample prior to step (b).

47(New). A method of claim 43 wherein the antibody agent used in one or more of steps (a) and (b) is immobilized.

48(New). A method of claim 45 wherein the antibody agent used in one or more of steps (a) and (b) is immobilized.

49(New). A method as claimed in claim 44 wherein the binding agent in one or both of steps (a) or (b) is immobilized.

50(New). A method as claimed in claim 46 wherein the binding agent in one or both of steps (a) or (b) is immobilized.

51(New). A method of removing a cellular form of a prion protein from a sample comprising the steps of:

- (a) exposing the sample to an agent selected from the group consisting of antibodies and binding agents as claimed in any one of claims 35, 37, 39 or 40; and
- (b) separating the sample from the agent which has bound the cellular form.

52(New). A method as claimed in any one of claims 38-40 wherein the sample consists of material selected from the group consisting of a bodily fluid and tissue.

53(New). A method as claimed in claim 52 wherein the sample is selected from one or more of the group consisting of blood, a blood component, cerebrospinal fluid, lymph, feces, urine, sputum, tissue from a lymph node, appendix, tonsil and spleen.

54(New). A method as claimed in claim 41 wherein the sample consists of material selected from the group consisting of a bodily fluid and tissue.

55(New). A method as claimed in claim 54 wherein the sample is selected from one or more of the group consisting of blood, a blood component, cerebrospinal fluid, lymph, feces, urine, sputum, tissue from a lymph node, appendix, tonsil and spleen.

56(New). A method as claimed in claim 42 wherein the sample consists of material selected from the group consisting of a bodily fluid and tissue.

57(New). A method as claimed in claim 56 wherein the sample is selected from one or more of the group consisting of blood, a blood component, cerebrospinal fluid, lymph, feces, urine, sputum, tissue from a lymph node, appendix, tonsil and spleen.

58(New). A method as claimed in claim 43 wherein the sample consists of material selected from the group consisting of a bodily fluid and tissue.

59(New). A method as claimed in claim 58 wherein the sample is selected from one or more of the group consisting of blood, a

blood component, cerebrospinal fluid, lymph, feces, urine, sputum, tissue from a lymph node, appendix, tonsil and spleen.

60(New). A method as claimed in claim 44 wherein the sample consists of material selected from the group consisting of a bodily fluid and tissue.

61(New). A method as claimed in claim 60 wherein the sample is selected from one or more of the group consisting of blood, a blood component, cerebrospinal fluid, lymph, feces, urine, sputum, tissue from a lymph node, appendix, tonsil and spleen.

62(New). A method as claimed in claim 51 wherein the sample consists of material selected from the group consisting of a bodily fluid and tissue.

63(New). A method as claimed in claim 62 wherein the sample is selected from one or more of the group consisting of blood, a blood component, cerebrospinal fluid, lymph, feces, urine, sputum, tissue from a lymph node, appendix, tonsil and spleen.

64(New). A peptide sequence of a cellular form of a prion protein as claimed in any one of claims 31-33 which exhibits stability when measured by hydrogen/deuterium amide exchange at pH 5.5.

65(New). A kit comprising an amount of one or more agents selected from the group consisting of antibodies and binding agents and detection means for carrying out a method of detecting a prion protein selected from the group consisting of cellular and non-cellular forms of prion protein.

REMARKS

Claims 1-30 have been canceled and new claims 31-65 are presently under consideration in this application.

Respectfully submitted,

NIKOLAI & MERSEREAU, P.A.



C. G. Mersereau
Registration No. 26205
820 International Centre
900 Second Avenue So.
Minneapolis, MN 55402
(612) 339-7461

BIOLOGICAL MATERIALS AND METHODS USEFUL IN THE
DIAGNOSIS AND TREATMENT OF DISEASES

The present invention relates to prion proteins.

5

Prions are infectious pathogens that differ from bacteria, fungi, parasites, viroids, and viruses, both with respect to their structure and with respect to the diseases that they cause. Molecular biological and structural studies of prions promise to open new vistas into fundamental mechanisms of cellular regulation and homeostasis not previously appreciated. Kuru, Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker syndrome (GSS) are all human neurodegenerative diseases that are caused by prions and are frequently transmissible to laboratory animals. Familial CJD and GSS are also genetic disorders. No effective therapy exists to prevent these fatal disorders².

15

In addition to the prion diseases of humans, disorders of animals are included in the group of known prion diseases. Scrapie of sheep and goats is the most studied of the prion diseases. Bovine spongiform encephalopathy (BSE) is thought to result from abnormal feeding practices. BSE threatens the beef industry of Great Britain and possibly other countries; the production of pharmaceuticals involving cattle is also of concern. Control of sheep scrapie in many countries is a persistent and vexing problem².

25

Since 1986, more than 170,000 cattle have developed BSE in Great Britain. Many investigators contend that BSE, often referred to as "mad cow disease", resulted from the feeding of dietary protein supplements

derived from rendered sheep offal infected with scrapie to cattle, a practice banned since 1988. It is thought that BSE will disappear with the cessation of feeding rendered meat and bone meal, as has been the case in kuru of humans, confined to the Fore region of New Guinea and once the most common cause of death among women and children. Kuru has almost disappeared with the cessation of ritualistic cannibalism.

Prion diseases are associated with the accumulation of a conformational isomer (PrP^{Sc}) of host-derived prion protein (PrP^{c}) with an increase in its β -sheet content¹. According to the protein-only hypothesis, PrP^{Sc} is the principal or sole component of transmissible prions². Although the structure of PrP^{c} has been determined³ and has been found to consist predominantly of α -helices, the insolubility of PrP^{Sc} , which is isolated from tissue in a highly aggregated state and which has a high β -sheet content, has precluded high-resolution structural analysis.

The detection of the disease-associated isoform of prion protein, PrP^{Sc} , in brain or other tissues from patients is thought to be diagnostic of prion disease. To distinguish PrP^{Sc} from its cellular precursor, PrP^{c} , requires either pre-treatment with proteinase K, which will completely digest PrP^{c} , but only removes a protease-sensitive N-terminal of PrP^{Sc} or, alternatively, would require an antibody which distinguished between PrP^{c} and PrP^{Sc} . Only one such selective antibody (Korth C. *et al.* 1997 *Nature* 390, 74-77) has yet been reported and appears to be able to selectively immunoprecipitate PrP^{Sc} . It is not clear as yet, however, whether this antibody offers any increase in diagnostic sensitivity over existing monoclonals. It is an IgM antibody and is likely to be of low affinity for PrP^{Sc} .

The invention also provides a peptide consisting of all, or a fragment or variant of an amino acid sequence of approximately ten residues which flanks the disulphide bond between Cys 179 and Cys 214 in one of the
5 PrP^C sequences shown in figure 5.

The disease-related isoform of PrP, PrP^{Sc}, is distinguished biochemically from the normal cellular isoform of the protein, PrP^C, by its partial resistance to digestion with the enzyme proteinase K.

10

By "cellular form" of a prion protein we include any form of a prion protein which does not exhibit partial resistance to digestion with proteinase K (PK).

15 By "partial resistance to digestion with proteinase K (PK)" we include the meaning that after incubation of 1 mg/ml of the protein in 10mM NaAcetate + 10mM Tris. Acetate, pH 8.0 with 0.5 µg/ml PK (based on the total digestion reaction volume) at 37°C for 30 minutes some protein can be shown to be undigested when subjected to SDS-PAGE as described
20 herein. Preferably, the majority of the protein is undigested.

Preferably, the non-cellular form displays resistance to digestion at increased concentrations of PK eg 5 µg/ml PK or more.

25 Non-cellular forms of a prion protein referred to herein exhibit partial resistance to digestion with PK and include PrP^{Sc} and the β-form as reported recently by Jackson *et al*, *Science* (March 1999) 283, pp 1933-1937.

The invention also provides a peptide of the invention for use in medicine, preferably in the prevention, treatment and/or diagnosis of a prion disease.

Preferably, the peptide sequence is selected from human, bovine or ovine
5 prion proteins, more preferably human prion protein.

It will be appreciated that peptides of the invention include variants, fragments and fusions that have interactions or activities which are substantially the same as those of the stable core sequence of PrP^C. A
10 relevant activity of the variants, fragments, or fusions of the invention is the ability to raise an antibody which binds preferentially to the cellular form of a prion protein, PrP^C, rather than the non-cellular form, such as PrP^{Sc}.

15 A "variant" will have a region which has at least 80% (preferably 85, 90, 95 or 99%) sequence identity with the stable core region of cellular human PrP^C sequence described herein or the corresponding region in the PrP^C of other species as measured by the Bestfit Program of the Wisconsin Sequence Analysis Package, version 8 for Unix. The percentage identity
20 may be calculated by reference to a region of at least 10 amino acids (preferably at least 20, 30, 40 or 45) of the candidate variant molecule, and the most similar region of equivalent length in the native region.

The percent identity may be determined, for example, by comparing
25 sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith

and Waterman (*Adv. Appl. Math* 2.482. 1981). The preferred default parameters for the GAP program include : (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Bribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986 as described by Schwartz and Dayhoff, eds, *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

10

The term "variants" is intended to embrace prion protein sequence variations between species, in particular, the known variations at residue numbers 184, 186, 203, 205, 215, 219 and 220 (see Figure 5).

15 A "fragment" comprises any peptide sequence which is antigenic ie capable of reacting with antibody a PrP^C specific and preferably is immunogenic ie capable of generating a PrP^C specific antibody response itself. The peptide sequence of the fragment will preferably comprise at least 45, preferably 6, 8, 10 or 12 or more amino acids of the native 176
20 to 221 PrP^C sequences described herein.

According to a further aspect of the invention there is provided a method of making an antibody comprising administering a cellular form (PrP^C) of a prion protein or a peptide as defined in accordance with the earlier
25 aspects of the invention, or a mixture of two or more of the peptides to an animal and collecting and optionally purifying the resulting antibody.

By "antibody" in accordance with the invention we include molecules which comprise or consist of antigen binding fragments of an antibody

including Fab, Fv, ScFv and dAb. We also include agents which incorporate such fragments as portions for targetting prion molecules and/or prokaryotic or eukaryotic cells or viruses which display such molecules.

5

According to this aspect of the invention, there is also provided a monoclonal antibody capable of distinguishing between the cellular form of a prion protein, PrP^C, and the non-cellular form of a prion protein (eg PrP^{Sc}) as defined in accordance with earlier aspects of the invention. Also
10 provided is a hybridoma cell capable of producing such a monoclonal antibody.

The invention also provides a method of making a binding agent capable of binding to a cellular form of a prion protein comprising exposing a
15 peptide of the invention to a sample whereby any binding agent can bind the peptide and collecting the binding agent so bound. Preferably, the binding agent is an antibody.

The invention also provides a binding agent, which is preferably
20 obtainable by the above method, which binds preferentially to a cellular form of a prion protein rather than a non-cellular form.

The invention also provides the use of an antibody or binding agent in a method of detecting a cellular form of a prion protein comprising exposing
25 a sample to the antibody or binding agent and detecting binding of the antibody or binding agent to the cellular form of a prion protein.

There is also provided the use of an antibody or binding agent of the invention in a method of detecting a non-cellular form of a prion protein

comprising a first step of exposing a sample to the antibody or binding agent; a second step of exposing the sample to an agent which binds the non-cellular form of a prion protein; and detecting binding the agent to the non-cellular form.

5

Preferably, the antibody or binding agent which has bound the cellular form is separated from the sample prior to the second step.

Conveniently, the antibody or binding agent in the first and/or second step is immobilised.

10

The invention also provides a method of removing a cellular form of a prion protein from a sample exposing the sample to an antibody or binding agent of the invention and separating the sample from the antibody or binding agent which has bound the cellular form.

15

The invention also provides a method of detecting antibodies in a sample, which antibodies bind preferentially to a non-cellular form of a prion protein rather than the cellular form, comprising exposing a peptide of the invention to the sample to permit binding of antibody to the peptide; and subsequently exposing the non-cellular form to the sample and detecting the binding of antibody to the non-cellular form. Optionally, the cellular and/or non-cellular form is immobilised before exposure to the sample.

20

The invention further provides a method of the invention for use in the prevention, treatment and/or diagnosis of a prion disease. Preferably, the method is for use in the diagnosis of the presence of a prion disease or a predisposition to such a disease.

25

The invention also provides a peptide of the invention, or an antibody or binding agent which binds preferentially to a cellular form of a prion protein rather than a non-cellular form of a prion protein, for use in medicine, preferably in the prevention, treatment and/or diagnosis of a prion disease.

The prion disease may be selected from one or more of the diseases affecting humans. Alternatively or additionally, the prion diseases are selected from one or more of the diseases which affect domestic farm animals such as cows, sheep and goats. Other prion diseases include transmissible mink encephalopathy; chronic wasting disease of mule deer and elk, bovine spongiform encephalopathy and, more recently, a whole series of new animal diseases that are thought to have arisen from their dietary exposure to the BSE agent. These include feline spongiform encephalopathy, affecting domestic cats and captive wild cats (such as cheetahs, pumas, ocelots, tigers) and spongiform encephalopathies of captive exotic ungulates (including kudu, nyala, gemsbok, eland).

The invention also provides a kit comprising means for carrying out the methods of the invention.

The invention also provides a kit useful for diagnosing a prion disease comprising a binding agent, preferably an antibody, which is capable of preferentially binding the cellular form rather than the non-cellular form, and means for detecting binding of the binding agent to the cellular form. Optionally, the binding agent is coupled to an inert support. Preferably, the means for detecting binding comprises a radioactive, enzymic or fluorescent label. Preferably, the kit further comprises a binding agent, preferably an antibody, which is capable of binding the non-cellular form of a prion

protein and means to detect binding of the binding agent to the non-cellular form.

Preferably, in the methods of the invention the sample comprises or
5 consists of a bodily tissue or fluid, which may be blood or a derivative of blood, ie a component such as plasma, or is derived from lymphoid tissue (such as tonsils, appendices, lymph nodes or spleen) or is cerebrospinal fluid, or faeces, urine or sputum, for example. The biological sample may be a tissue sample eg a biopsy tissue sample.

10

By “preferentially” according to the various aspects of the invention we include the meaning that the ratio of noncellular/cellular binding may be 45/55, 25/75, more preferably, 10/90, 5/95, 1/99 or substantially 0/100.

15 Preferred non-limiting embodiments of the invention will now be described by way of example with reference to the following figures in which:

Figure 1

20

Aromatic CD measured at 290 nm is shown as open triangles, overlaid with amide CD measured at 222 nm, shown as open circles. The lines superimposed upon the data are fits to the function:

25

$$\alpha_N = (K_{(N/U)} \cdot \exp(m \cdot D)) / (1 + K_{(N/U)} \cdot \exp(m \cdot D))$$

where m represents the sensitivity of the unfolding transition to denaturant and D is the denaturant activity ²⁴.

Figure 2

- (a) Amide protection factors ($K_{\text{ex}}/K_{\text{int}}$) of the residues with quantifiable protection in human PrP^C (HuPrP). The protection factor corresponding to the equilibrium constant between the native (N) and unfolded (U) states is plotted as a horizontal dashed line ($\log_{10}K_{(N/U)}$). Regions of highest protection (greater than $K_{(N/U)}$) are in close proximity to the disulphide bond between helices II and III (see figure 4).
- (b) C^α chemical shift deviations from random coil values for residues not preceeding proline in human PrP^C ²⁵. A very similar C^α chemical shift profile is observed in hamster PrP^C (sHaPrP)²⁶ strongly suggesting that both proteins have very similar global folds.
- (c) Chemical Shift Index (CSI) of human PrP^C calculated by the program CSI²² using C^α/C^β/C' resonances, showing regions predicted to be β-sheet (values above line) and α-helix (values below line). As found in sHaPrP, the first strand of the β-sheet (residues 129-131) observed in the solution structure is not identified by the CSI²⁶, however residues 129 and 130 of HuPrP have a C^α chemical shifts consistent with an extended conformation (see (b)).
- (d) Secondary structure elements of hamster (sHaPrP) and mouse (moPrP) PrP^C, in their respective NMR structures^{9,11,27}. α-helices are represented by cylinders and the β-sheet strands by arrows. The three main α-helices are labelled I-III.

Figure 3

The decay curves of three representative amide protons within human PrP are shown: Val209 (open circles), Val210 (open squares) and Gln212 (open triangles). Superimposed on the data are fits to the first order rate equation, $I_t = I_0 \cdot e^{-(k \cdot t)}$, where I_t is the relative intensity at time t , I_0 is the initial intensity, t is time in secs and k is the first order rate constant.

Figure 4

10

Stereoview of protected amide protons in human PrP^C displayed on the backbone conformation of mouse PrP^C ⁹ (moPrP). (The human form of the protein differs from mouse by a number of point mutations which can be modelled onto the moPrP NMR structure with very little effect on its backbone conformation (Unpublished data)). Residues 124-226 are displayed, with β -strands coloured blue and α -helices red. The ends of the helices are marked with roman numerals, with the numeral at the C-terminal end marked with an apostrophe. The disulphide bond between Cys179 and Cys214 is marked by a yellow dashed line between the C $^{\alpha}$ atom of those residues. The C $^{\alpha}$ atoms of significantly protected amide groups are shown as spacefilled spheres with the radius of each proportional to \log_{10} (Protection Factor). This figure was produced using MidasPlus^{28,29}.

Figure 5

Known prion protein sequences from various mammalian species, using the single letter code for amino acids as follows:

The ligated pTrcHisB/*PRNP* construct was used to transform the *E. coli* host strain BL21 (DE3) (Novagen), genotype F' *ompT hsdS_B (r_Bm_B) gal dcm* (DE3) which was then plated onto Luria-Bertoni (LB) agar plates
 5 containing 100µg/ml carbenicillin. Following growth overnight at 37°C single colonies were picked and used to inoculate 10 x 10ml of LB broth containing 100µg/ml carbenicillin. This culture was grown overnight at 37°C with vigorous shaking. The 10ml cultures were used as inocula for 10 x 1 litre of LB broth containing 100µg/ml carbenicillin which had been
 10 pre-warmed to 37°C. Growth at 37°C with vigorous shaking was allowed to progress until the culture reached an OD₆₀₀ of 0.6. Expression was then induced by addition of isopropyl-β-D-galactopyranoside to a final concentration of 1mM and the culture resupplemented with carbenicillin to a level of 100µg/ml. Following 4 hours of induced growth the cells were
 15 harvested by centrifugation at 8,500 rpm for 10 minutes.

Extraction, Refolding and Purification of Recombinant Human PrP

The cell pellet was resuspended in 50ml of lysis buffer (50mM Tris. Cl
 20 pH 8.0, 200mM NaCl, 0.1% Triton X100, 10µg/ml DNase 1, 10µg/ml lysozyme) and disrupted by sonication in 1 minute bursts for a total of 5 minutes. Centrifugation at 9,600 rpm for 30 minutes pelleted all the insoluble material and the supernatant was discarded. The pellet was then washed twice by resuspension in 50ml of lysis buffer with centrifugation
 25 at 7,500 rpm for 5 minutes between each wash. Solubilisation of protein in the pellet was performed by resuspension in 50ml of 50mM Tris. Cl, 6M GuHCl, 100mM DTT pH 8.0. Cell debris and insoluble material was removed by centrifugation at 9,600rpm for 30 minutes. The supernatant was clarified by passage through a 0.2µm filter and loaded onto a 20ml

Ni-NTA-Sepharose (Quiagen) column pre-equilibrated with 50mM Tris.Cl, 6M GuHCl pH 8.0.

After washing the column with the above buffer, bound protein was eluted
5 with a 15 column volume linear gradient of 0mM to 300mM imidazole in
loading buffer. Recombinant PrP eluted at 185mM imidazole. Eluted
fractions were pooled and oxidation of disulphides was achieved by
vigorous stirring in the presence of 1 μ M CuSO₄ and dissolved atmospheric
oxygen for 16 hours. PrP containing oxidised disulphides was separated
10 from reduced protein using reverse phase chromatography on an RP304-
C4 column. The protein was loaded in 50mM Tris.Cl, 6M GuHCl pH
8.0, washed with ddH₂O + 0.1% trifluoroacetic acid (TFA) and eluted
with a linear gradient of 15% to 60% acetonitrile + 0.09% TFA. Human
PrP emerged as two major peaks; oxidised protein at 40% acetonitrile and
15 a second peak containing reduced PrP eluted at 45% acetonitrile. The
oxidised peak fractions were pooled and neutralised by the addition of 1M
Tris.Cl pH 8.0 to a final concentration of 100mM and saturated
ammonium sulphate added to a final concentration of 70%. Precipitated
PrP accumulated at the interface between organic and aqueous phases and
20 was removed to a separate container. The protein was solubilised in a
minimal volume of 50mM Tris.Cl, 6M GuHCl pH 8.0 and then diluted
rapidly to a protein concentration of 1mg/ml and dialysed for 16 hours
against 50mM Tris.Cl pH 8.0 with a buffer change after 8 hours.
Following dialysis the N-terminal fusion peptide was removed by addition
25 of enterokinase at 1unit/3mg protein. Cleavage was allowed to occur at
37°C for 14 hours and terminated by the addition of "protease complete"
(Boehringer Mannheim Corp).

Final purification was carried out by applying the protein material to a 10ml S-Sepharose FastFlow column equilibrated with 25mM Tris.Cl pH 7.0 and following a 5 column volume wash with the same buffer, protein was eluted with a 10 column volume linear gradient of 0mM to 300mM NaCl. Recombinant PrP lacking the N-terminal fusion peptide eluted at 150mM whilst uncleaved material remained bound until 250mM NaCl. Eluted fractions were concentrated in an Amicon cell with a 10kDa cut off membrane and then dialysed overnight against 25mM Tris.Cl pH 7.0, 0.02% NaAzide containing a small amount of activated charcoal. Sucrose was added to 5% w/v and the protein snap frozen in liquid nitrogen for long term storage at -80°C.

2. *NMR spectroscopy*

NMR spectra were acquired at 303K on 12 mg/ml $^{13}\text{C}/^{15}\text{N}$ -labelled sample in 20 mM sodium acetate- d_3 , 2mM Sodium Azide, pH 5.5 (10%(v/v) D_2O) using Bruker DRX-500 and DRX-600 spectrometers. Backbone resonances (H^{N} ; N; C^{α} ; C' ; C^{β}) were assigned using a suite of triple-resonance NMR experiments^{18,18-21}. Almost complete backbone assignments were determined, the exceptions being residues 164-172, which form an ill-defined loop in the mouse PrP^C NMR structure^{9,11}. C^{α} ; C' ; C^{β} chemical shifts were used to calculate the chemical shift index (CSI) profile of human PrP^C²². NMR data were processed and analysed on Silicon Graphics Workstations using Felix 97 software (MSI Corp).

3. *Amide exchange*

Amide exchange was initiated by diluting the $^{13}\text{C}/^{15}\text{N}$ -labelled human PrP^C sample with an equal volume of 20 mM sodium acetate- d_3 , 2 mM Sodium

Azide, pH 5.5 dissolved in 100% D₂O. The sample was equilibrated at 303K for 5 mins in a Bruker DRX-600 spectrometer and amide exchange rate constants (K_{ex}) determined from a series ¹H-¹⁵N HSQC spectra. These were used to determine protection factors (K_{ex}/K_{int}) for observable amides using intrinsic exchange rates (K_{int})²³. Acquisition of the first experiment began ~5 mins after mixing, setting a lower limit on the detection of protection factors of approximately 10.

4. *Equilibrium denaturation data*

10

The amide C.D. absorption of 5 μ M PrP^C dissolved in 20mM sodium acetate-d₃, 2 mM Sodium Azide, pH 5.5 55% D₂O was recorded in varying concentrations of GuHCl at 222 nm on a Jobin-Yvon CD6 spectropolarimeter through 1cm light path with an integration time of 120 sec. The ellipticity signal (O) was converted to proportion of molecules in the native state α_N according to the relationship $\alpha_N = (O - O_U) / (O_N - O_U)$, where O_U and O_N are the ellipticity signals for the unfolded and native states respectively. Similarly the aromatic C.D. absorption response of 50 μ M PrP was measured at 290 nm through a 1cm light path at increasing denaturant concentration.

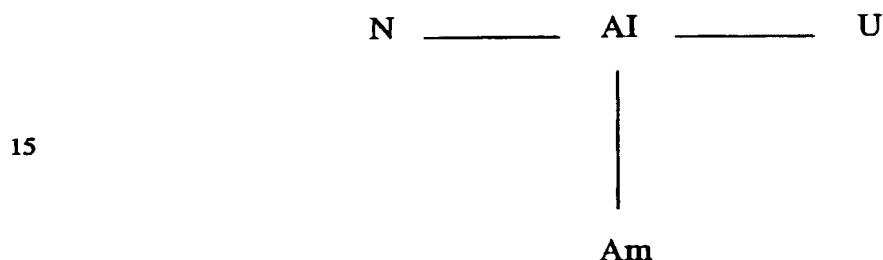
20

5. *Results*

The accumulation of insoluble PrP^{Sc} by recruitment of the native, cellular isoform of the prion protein (PrP^C) is a characteristic of spongiform encephalopathies such as Scrapie in sheep, BSE in cows and CJD in humans¹. It appears that the disease process can be triggered by inherited mutations in the encoding gene^{2,3}, by infection with tissue containing PrP^{Sc} or by a rare spontaneous event which gives rise to sporadic cases. *In vitro*

25

studies of prion proteins show that the chain can adopt a variety of folds depending on solution conditions^{4,5}. The native, cellular form (PrP^C) is capable of being switched between the largely α -helical conformation, characteristic of this state, to a variety of other structures by changing
 5 temperature, pH and/or redox conditions⁴. Most of these alternative states are dominated by β -sheet and form insoluble aggregates. Such observations have led to the proposal that the prion protein shows unusual conformations, enriched in β -sheet, which are in equilibrium with the native PrP^C state. In turn these alternative conformers, or a subset
 10 thereof, can then irreversibly associate to form amyloid rods⁶. This process is described most simply by the following scheme:



Where N is the native state, AI is an amyloidogenic folding intermediate
 20 and Am is irreversibly aggregated amyloid material. In this model, formation of the prion amyloid can be promoted by mutations which increase the population of the amyloidogenic intermediate and by the introduction of stable amyloid material which can then recruit such an intermediate. This type of mechanism for the conversion of PrP^C to PrP^{Sc}
 25 can, to some degree, be tested by comparing the free energy of unfolding of the PrP^C protein with its local hydrogen exchange properties.

Shown in figure 1 are equilibrium denaturation profiles of human PrP^C at pH 5.5 and 55% D₂O; a condition chosen to match the hydrogen exchange

measurements described later. The amide and aromatic CD profiles show that the molecule denatures in a single transition to a state that retains some degree of backbone and aromatic organisation (~30% of the native signal). Analysis of the amide and aromatic transitions renders values of
 5 1.5×10^3 and 1.7×10^3 respectively for the equilibrium constant $K_{(N/U)}$ in the absence of denaturant.

The fact that there is no populated intermediate state during equilibrium denaturation in these conditions does not rule out the existence of a folding
 10 intermediate (I) which, in water, is more stable than the unfolded state (U). Indeed, in most proteins such folding intermediates are only measurably populated in kinetic experiments where the unfolded state, in a denaturing solvent, is rapidly transferred to strong folding conditions. The folding reaction can then be written:

15



If $K_{(U/I)}$ and $K_{(N/I)}$ are greater than 1 and the 'U <-> I' equilibrium is fast (as with most proteins) then the intermediate, despite being more stable
 20 than the unfolded state, is only seen as a transient species. Alternatively, or additionally, the native state (N) may be in equilibrium with a partially unfolded form (PUF)⁷ so that the system can be written:



25

The question we wish to address is whether it is likely that PrP^{Sc}, an aggregated β -sheet form of the prion protein, is generated from folding intermediates or partially unfolded forms which are rich in β -sheet structure and capable of forming strong intermolecular interactions. This

type of mechanism has recently been proposed for the formation of lysozyme amyloid⁸.

One way of examining this question is to measure the rate of hydrogen/deuterium exchange by NMR. This enables identification of those regions of the hydrogen-bonded backbone which remain stable until the molecule is fully unfolded. The observed rate of H/D exchange of an amide proton (k_{ex}) is governed by its intrinsic exchange rate when exposed to the solvent (k_{int}), and the rates of closing (k_c) and opening (k_o) of the hydrogen bond. The general expression relating these rate constants is

$$k_{ex} = (k_o \cdot k_{int}) / (k_c + k_{int})$$

In conditions where $k_c > k_{int}$ (often termed the EX2 condition) the relationship reduces to $k_{ex} = (k_o/k_c) \cdot k_{int}$ so that exchange data can be used to map the local equilibrium distribution between open and closed backbone hydrogen bonds. The average rate constant for intrinsic exchange at pH 5.5 is about 0.1 sec^{-1} , yet the native state signal in both the amide and aromatic regions of the C.D spectrum is gained within 20 milliseconds of initiation of folding at pH 5.5 55% D_2O (with a residual concentration of GuHCl of 0.37 M). Hence, we can be confident the closing rate associated with hydrogen bond formation during folding ($> 50 \text{ sec}^{-1}$) is comfortably larger than the intrinsic exchange rate.

In these circumstances, the equilibrium constant for hydrogen-bond formation $k_{c/o}$ is defined by the ratio k_c/k_o which is equivalent to the protection factor (P) where $P = k_{int}/k_{ex}$. Having determined the equilibrium constant $K_{(N/U)}$ by the denaturation method and assuming that all backbone amide protons are free to exchange in the unfolded state, it follows that

any regions of the molecule which have a protection factor (P) equal to $K_{(N/U)}$ can only exchange in the unfolded state. In other words, such measurements delineate sub-structures which are in a protected, hydrogen-bonded environment in any intermediate or partially folded state which is
5 more stable than the unfolded chain.

To perform this study, the backbone amide protons were first assigned as described in the legend to figure 2 and the carbon chemical shifts show that the backbone conformation of the human protein is essentially the
10 same as those of mouse^{9,10} and hamster PrP^C¹¹, consisting of three α -helices and short distorted β -sheet (see figure 2b, figure 4).

When the molecule was subjected to hydrogen/deuterium exchange at pH 5.5 only 23 non-overlapping amide protons were protected to a
15 measurable level in the experiment, ie with protection factors greater than 200 (see Figure 2a). These were located predominantly in the three helical segments of the structure which associate together to form the core of the molecule. Protection factors for these 23 residues show the stability of these sub-structures to be equivalent to the free energy change for
20 unfolding of human PrP^C. This type of behaviour resembles that of conventional proteins (eg barnase¹², CI-2¹³, N-PGK¹⁴ and Staphylococcal nuclease¹⁵) in that a subset of core residues can only exchange in the fully unfolded state. The only residues in these protected regions which are exceptional are those at or adjacent to the cysteine bridge. These have
25 protection factors which are anomalously high, presumably owing to residual local structure in the unfolded state, a property which is consistent with the incomplete loss of CD signals in the denatured state.

The pattern of protection is so well correlated with the core structure of the native PrP^C conformation that it is difficult to conceive of a grossly different topology, for instance one dominated by β -sheet, that would be consistent with this pattern. Hence, from these data, we conclude that

5 there are no structured species present, either folding intermediates or alternatively folded states, in which this three-helical core region is unfolded or significantly rearranged. If such species are present their combined population must be less than that of the unfolded state. In principle, this observation can be interpreted in one of two ways. Firstly,

10 if prion amyloid formation occurs by assembly of a partially folded intermediate then this intermediate must retain these α -helical regions intact. Secondly, if the formation of amyloid states requires the disruption of this core structure, then the molecule must pass through a highly unfolded state before adopting an alternative fold capable of self-assembly.

15 Since, in all cases where secondary structure content has been assessed, the amyloid aggregate or amyloidogenic precursor states have been shown to be dominated by β -structure, the first mechanism appears improbable. Elimination of this route leads to the conclusion that complete or near-complete unfolding must precede rearrangement to the amyloidogenic

20 intermediate.

While it initially seems unlikely that complete disruption of the native fold is prerequisite for forming a misfolded, multimolecular state, there is a precedent for this type of behaviour. The N-terminal domain of the cell

25 surface receptor, CD2, forms interpenetrated dimers, trimers and tetramers which are misfolded forms of the normally monomeric state¹⁶. Experiments elucidating the folding pathway of the monomer show that it attains the native state via a topologically well-organised transient intermediate¹⁷. However, the degree of inter-penetration of the

polypeptide chain in these multimeric states means that the monomeric precursor must be much more unfolded than the transient intermediate.

5 Interestingly, in the case of amyloidogenic variants of lysozyme⁸ it was proposed that fibril assembly occurred through the interaction of molecules that had the conformational properties of folding intermediates and that the mutations increased the population of these states, so promoting ordered aggregation. However, in the case of the prion diseases, in which native PrP^C monomers are recruited into PrP^{Sc} fibrils, it
10 appears that the involvement of a folding intermediate or partially unfolded form is unlikely.

6. *Digestion with proteinase K*

15

Prion protein is subjected to digestion with varying concentrations of proteinase K (BDH) at 37°C for 1 hr. Protein is digested at a concentration of 1m/ml in 10mM NaAcetate + 10mM Tris. Acetate pH 8.0. Digestion is terminated by the addition of Pefablock (Boehringer
20 Mannheim Corp.) to a final concentration of 1mM. Following the addition of Pefabloc samples are heated to 100°C for 5 mins in the presence of SDS loading buffer. Aliquots of 20µl are subjected to SDS-PAGE and the gels stained with Coomassie brilliant blue.

25 7. *Antibody production methods*

Methods for purification of antigens and antibodies are described in Scopes, R.K. (1993) *Protein purification* 3rd Edition. Publisher - Springer Verlag. ISBN 0-387-94072-3 and 3-540-94072-3. The disclosure

of that reference, especially chapters 7 and 9, is incorporated herein by reference.

A monoclonal antibody which binds to a non-cellular form of a prion protein, namely PrP^{Sc}, is disclosed by Korth *et al*, 1997 *Nature* 390, 74-77.

Methods of raising polyclonal and monoclonal antibodies to a non-cellular (PrP^{Sc}) forms of prion proteins are described by Serban *et al*, (1990) *Neurology* 40, 110-117 and Kascsak *et al*, (1987) *J Virol* 61:12, 3688-3698.

The disclosures of the above documents are incorporated herein by reference.

The above and other methods can also be used to raise antibodies (polyclonal and monoclonal) to a non-cellular β -form of a prion protein which was described recently by Jackson *et al*, *Science* (March 1999) 283, pp 1935-1937.

Antibodies may be produced in a number of ways.

1 The prion protein or peptide is purified. The immunisation animal may be a "knock-out" mouse, with no prion protein at all. For monoclonal antibodies the animal is normally a mouse; for polyclonal, a rabbit or goat.

2. Raise antibodies to the antigen. For polyclonal antibodies, this is simply a matter of injecting suitably prepared sample into the animal at intervals, and testing its serum for the presence of antibodies (for

details, see Dunbar, B.S. & Schwoebel, E.D. (1990) Preparation of polyclonal antibodies. *Methods Enzymol.* 182, 663-670). But it is essential that the antigen (ie. the protein of interest) be as pure as possible. For monoclonal antibodies, the purity of the antigen is relatively unimportant if the screening procedure to detect suitable clones uses a bioassay.

Antibodies can also be produced by molecular biology techniques, with expression in bacterial or other heterologous host cells (Chiswell, D.J. & McCafferty, J. (1992) Phage antibodies: will new "coli-clonal" antibodies replace monoclonal antibodies?" *Trends Biotechnol.* 10, 80-84). The purification method to be adopted will depend on the source material (serum, cell culture, bacterial expression culture, etc.) and the purpose of the purification (research, diagnostic investigation, commercial production). The major methods are as follows:

1. *Ammonium sulphate precipitation.* The γ -globulins precipitate at a lower concentration than most other proteins, and a concentration of 33% saturation is sufficient. Either dissolve in 200g ammonium sulphate per litre of serum, or add 0.5 vol of saturated ammonium sulphate. Stir for 30 minutes, then collect the γ -globulin fraction by centrifugation, redissolve in an appropriate buffer, and remove excess ammonium sulphate by dialysis or gel filtration.
2. *Polyethylene glycol precipitation.* The low solubility of γ -globulins can also be exploited using PEG. Add 0.1 vol of a 50% solution of PEG 6,000 to the serum, stir for 30 minutes and collect the γ -globulins by centrifugation. Redissolve the precipitate in an appropriate buffer, and remove excess PEG by gel filtration on a

column that fractionates in a range with a minimum around 6,000 Da.

3. *Isoelectric precipitation.* This is particularly suited for IgM
5 molecules, and the precise conditions will depend on the exact properties of the antibody being produced.
4. *Ion-exchange chromatography.* Whereas most serum proteins have low isoelectric points, γ -globulins are isoelectric around neutrality,
10 depending on the exact properties of the antibody being produced. Adsorption to cation exchangers in a buffer of around pH 6 has been used successfully, with elution with a salt gradient, or even standard saline solution to allow immediate therapeutic use.
- 15 5. *Hydrophobic chromatography.* The low solubility of γ -globulins reflects their relatively hydrophobic character. In the presence of sodium or ammonium sulphate, they bind to many hydrophobic adsorbents, such as "T-gel" which consists of β -mercaptoethanol coupled to divinyl sulphone-activated agarose.
- 20 6. *Affinity adsorbents.* *Staphylococcus aureus* Outer coat protein, known as Protein A, is isolated from the bacterial cells, and it interacts very specifically and strongly with the invariant region (F_c) of immunoglobulins (Kessler, S.W. (1975) *Rapid isolation of*
25 *antigens from cells with a staphylococcal protein A-antibody absorbent: Parameters of the interaction of antibody-antigen complexes with protein A. J Immunol.* 115, 1617-1624. Protein A has been cloned, and is available in many different forms, but the most useful is as an affinity column: Protein A coupled to agarose.

A mixture containing immunoglobulins is passed through the column, and only the immunoglobulins adsorb. Elution is carried out by lowering the pH; different types of IgG elute at different pHs, and so some trials will be needed each time. The differences in the immunoglobulins in this case are not due so much to the antibody specificity, but due to different types of F_c region. Each animal species produces several forms of heavy chain varying in the F_c region; for instance, mouse immunoglobulins include subclasses IgG₁, IgG_{2a}, and IgG₃ all of which behave differently on elution from Protein A.

Some γ -globulins do not bind well to Protein A. An alternative, Protein G from a *Streptococcus* sp., can be used. This is more satisfactory with immunoglobulins from farm animals such as sheep, goats and cattle, as well as with certain subclasses of mouse and rabbit IgGs.

The most specific affinity adsorbent is the antigen itself. The process of purifying an antibody on an antigen adsorbent is essentially the same as purifying the antigen on an antibody adsorbent. The antigen is coupled to the activated matrix, and the antibody-containing sample applied. Elution requires a process for weakening the antibody-antigen complex. This is particularly useful for purifying a specific antibody from a polyclonal mixture.

Monoclonal antibodies (MAbs) can be prepared to most antigens. The antigen-binding portion may be a part of an antibody (for example a Fab fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]). Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in

"Monoclonal Antibodies: A manual of techniques", H Zola (CRC Press, 1988) and in *"Monoclonal Hybridoma Antibodies: Techniques and Applications"*, J G R Hurrell (CRC Press, 1982).

- 5 Chimaeric antibodies are discussed by Neuberger *et al* (1988, *8th International Biotechnology Symposium Part 2*, 792-799).

Suitably prepared non-human antibodies can be "humanized" in known ways, for example by inserting the CDR regions of mouse antibodies into
10 the framework of human antibodies.

The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation"
15 of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parental antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855).

- 20 That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**,
25 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in

the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* 349, 293-299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner
5 domains are linked via a flexible oligopeptide.

The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue.
10 Effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

15 Whole antibodies, and $F(ab')_2$ fragments are "bivalent". By "bivalent" we mean that the said antibodies and $F(ab')_2$ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.

20 A CDR-grafted antibody may be produced having at least one chain wherein the framework regions are predominantly derived from a first antibody (acceptor) and at least one CDR is derived from a second antibody (donor), the CDR-grafted antibody being capable of binding to the β -form PrP antigen.

25

The CDR-grafted chain may have two or all three CDRs derived from the donor antibody.

Advantageously, in the CDR-grafted chain, the or each CDR comprises a composite CDR comprising all the residues from the CDR and all the residues in the corresponding hypervariable region of the donor antibody.

- 5 Preferably, at least one residue in the framework regions of the CDR-grafted chain has been altered so that it corresponds to the equivalent residue in the antibody, and the framework regions of the CDR-grafted chain are derived from a human antibody.
- 10 Advantageously, the framework regions of the CDR-grafted chain are derived from a human Ig heavy chain. For such heavy chains, it is preferred that residue 35 in the heavy chain framework regions be altered so that it corresponds to the equivalent residue in the donor antibody.
- 15 Suitably, for such heavy chains, at least one composite CDR comprising residues 26 to 35, 50 to 65 or 95 to 102 respectively is grafted onto the human framework. It will be appreciated in this case that residue 35 will already correspond to the equivalent residue in the donor antibody.
- 20 Preferably, residues 23, 24 and 49 in such heavy chains correspond to the equivalent residues in the antibody. It is more preferred that residues 6, 23, 24, 48 and 49 in such heavy chains correspond to the donor antibody in equivalent residue positions. If desired, residues 71, 73 and 79 can also so correspond.
- 25 To further optimise affinity, any one or any combination of residues 57, 58, 60, 88 and 91 may correspond to the equivalent residue in the donor antibody.

The heavy chain may be derived from the human KOL heavy chain. However, it may also be derived from the human NEWM or EU heavy chain.

- 5 Alternatively, the framework regions of the CDR-grafted chain may be derived from a human kappa or lambda light chain. For such a light chain, advantageously at least one composite CDR comprising residues 24 to 34, 50 to 56 or 89 to 97 respectively is grafted onto the human framework. Preferably, residue 49 also corresponds to the equivalent residue in the
10 donor antibody.

To further optimise affinity, it is preferable to ensure that residues 49 and 89 correspond to the equivalent residues in the donor antibody. It may also be desirable to select equivalent donor residues that form salt bridges.

15

The light chain is preferably derived from the human REI light chain. However, it may also be derived from the human EU light chain.

- Preferably, the CDR-grafted antibody comprises a light chain and a heavy
20 chain, one or, preferably, both of which have been CDR-grafted in accordance with the principles set out above for the individual light and heavy chains.

- It is advantageous that all three CDRs on the heavy chain are altered and
25 that minimal alteration is made to the light chain. It may be possible to alter none, one or two of the light chain CDRs and still retain binding affinity at a reasonable level.

It will be appreciated that in some cases, for both heavy and light chains, the donor and acceptor residues may be identical at a particular position and thus no change of acceptor framework residue will be required.

- 5 It will also be appreciated that in order to retain as far as possible the human nature of the CDR-grafted antibody, as few residue changes as possible should be made. It is envisaged that in many cases, it will not be necessary to change more than the CDRs and a small number of framework residues. Only in exceptional cases will it be necessary to change a larger number of
10 framework residues.

Preferably, the CDR-grafted antibody is a complete Ig, for example of isotype IgG₁, or IgG₂, IgG₃ or IgM.

- 15 If desired, one or more residues in the constant domains of the Ig may be altered in order to alter the effector functions of the constant domains.

Preferably, the CDR-grafted antibody has an affinity for the β -form PrP antigen of between about $10^5 \cdot \text{M}^{-1}$ to about $10^{12} \cdot \text{M}^{-1}$, more preferably at least
20 $10^8 \cdot \text{M}^{-1}$.

Advantageously, the or each CDR is derived from a mammalian antibody and preferably is derived from a murine MAb.

- 25 Suitably, the CDR-grafted antibody is produced by use of recombinant DNA technology.

A further method for producing a CDR-grafted antibody comprises providing a first DNA sequence, encoding a first antibody chain in which

the framework regions are predominantly derived from a first antibody (acceptor) and at least one CDR is derived from a second antibody (acceptor), under the control of suitable upstream and downstream elements; transforming a host cell with the first DNA sequence; and culturing the transformed host cell so that a CDR-grafted antibody is produced.

Preferably, the method further comprises: providing a second DNA sequence, encoding a second antibody chain complementary to the first chain, under the control of suitable upstream and downstream elements; and
10 transforming the host cell with both the first and second DNA sequences.

Advantageously, the second DNA sequence encodes a second antibody chain in which the framework regions are predominantly derived from a first antibody (acceptor) and at least one CDR is derived from the second antibody (donor).

The first and second DNA sequences may be present on the same vector. In this case, the sequences may be under the control of the same or different upstream and/or downstream elements.

Alternatively, the first and second DNA sequences may be present on different vectors.

A nucleotide sequence may be formed which encodes an antibody chain in which the framework regions are predominantly derived from a first antibody (acceptor) and at least one CDR is derived from a second antibody (donor), the antibody chain being capable of forming a CDR-grafted antibody.

The CDR-grafted antibodies may be produced by a variety of techniques, with expression in transfected cells, such as yeast, insect, CHO or myeloma cells, being preferred. Most preferably, the host cell is a CHO host cell.

- 5 To design a CDR-grafted antibody, it is first necessary to ascertain the variable domain sequence of an antibody having the desired binding properties. Suitable source cells for such DNA sequences include avian, mammalian or other vertebrate sources such as chickens, mice, rats and rabbits, and preferably mice. The variable domain sequences (V_H and V_L)
10 may be determined from heavy and light chain cDNA, synthesized from the respective mRNA by techniques generally known to the art. The hypervariable regions may then be determined using the Kabat method (Wu and Kabat, J. (1970) *J. Exp. Med.* 132, 211). The CDRs may be determined by structural analysis using X-ray crystallography or molecular
15 modelling techniques. A composite CDR may then be defined as containing all the residues in one CDR and all the residues in the corresponding hypervariable region. These composite CDRs along with certain select residues from the framework region are preferably transferred as the "antigen binding sites", while the remainder of the antibody, such as the
20 heavy and light chain constant domains and remaining framework regions, may be based on human antibodies of different classes. Constant domains may be selected to have desired effector functions appropriate to the intended use of the antibody so constructed. For example, human IgG isotypes, IgG₁ and IgG₃ are effective for complement fixation and cell
25 mediated lysis. For other purposes other isotypes, such as IgG₂ and IgG₄, or other classes, such as IgM and IgE, may be more suitable.

For human therapy, it is particularly desirable to use human isotypes, to minimise antiglobulin responses during therapy. Human constant domain

DNA sequences, preferably in conjunction with their variable domain framework bases can be prepared in accordance with well-known procedures. An example of this is CAMPATH 1H available from Glaxo Wellcome.

5

Certain CDR-grafted antibodies are provided which contain select alterations to the human-like framework region (in other words, outside of the CDRs of the variable domains), resulting in a CDR-grafted antibody with satisfactory binding affinity. Such binding affinity is preferably from
10 about $10^5.M^{-1}$ to about $10^{12}.M^{-1}$ and is more preferably at least about $10^8.M^{-1}$.

In constructing the CDR-grafted antibodies, the V_H and/or V_L gene segments may be altered by mutagenesis. One skilled in the art will also
15 understand that various other nucleotides coding for amino acid residues or sequences contained in the Fc portion or other areas of the antibody may be altered in like manner (see, for example, PCT/US89/00297).

Exemplary techniques include the addition, deletion or nonconservative
20 substitution of a limited number of various nucleotides or the conservative substitution of many nucleotides, provided that the proper reading frame is maintained.

Substitutions, deletions, insertions or any subcombination may be used to
25 arrive at a final construct. Since there are 64 possible codon sequences but only twenty known amino acids, the genetic code is degenerate in the sense that different codons may yield the same amino acid. Thus there is at least one codon for each amino acid, ie each codon yields a single amino acid and no other. It will be apparent that during translation, the proper reading

frame must be maintained in order to obtain the proper amino acid sequence in the polypeptide ultimately produced.

Techniques for additions, deletions or substitutions at predetermined amino acid sites having a known sequence are well known. Exemplary techniques include oligonucleotide-mediated site-directed mutagenesis and the polymerase chain reaction.

Oligonucleotide site-directed mutagenesis in essence involves hybridizing an oligonucleotide coding for a desired mutation with a single strand of DNA containing the region to be mutated and using the single strand as a template for extension of the oligonucleotide to produce a strand containing the mutation. This technique, in various forms, is described in Zoller and Smith (1982) *Nucl. Acids Res.* **10**, 6487.

15

8. *Raising an antibody response in a patient*

Active immunisation of the patient is preferred. In this approach, one or more PrP proteins or peptides are prepared in an immunogenic formulation containing suitable adjuvants and carriers and administered to the patient. Suitable adjuvants include Freund's complete or incomplete adjuvant, muramyl dipeptide, the "Iscoms" of EP 109 942, EP 180 564 and EP 231 039, aluminium hydroxide, saponin, DEAE-dextran, neutral oils (such as miglyol), vegetable oils (such as arachis oil), liposomes, Pluronic polyols or the Ribi adjuvant system (see, for example GB-A-2 189 141). "Pluronic" is a Registered Trade Mark.

25

Skilled persons will appreciate that purification of the cellular form and/or cellular-form binding agents, especially antibodies, can be accomplished

by conventional techniques such as affinity chromatography. By "cellular form binding agent" we include any agent which is able to binds preferentially the cellular form rather than the non-cellular form of a prion protein. The binding agent is preferably an antibody or antigen binding
5 fragment thereof such a Fab, Fv, ScFv and Ab, but it may also be any other ligand which exhibits the preferential binding characteristic mentioned above.

Affinity chromatography is described in Scopes, R. K. (1993) *Protein*
10 *Purification: principles and practice* 3rd Ed. Springer-Verlag, New York, ISBN 0-387-44072-3, 3-540-94072-3. (See chapters 7 and 9 in particular).

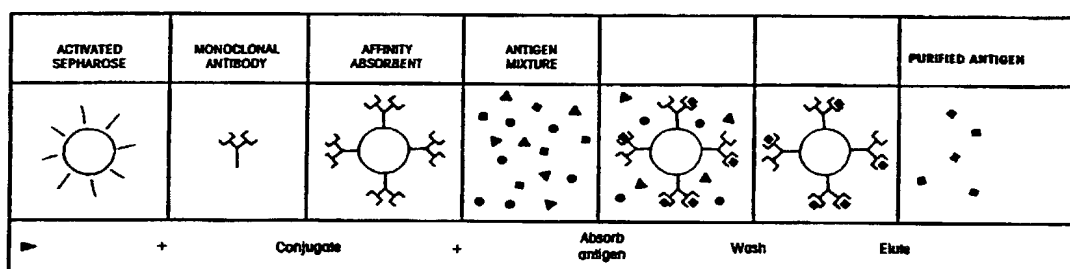
Further information on the above affinity chromatography techniques and
15 the immunoassay of antigen and antibody is provided by Roitt (1991) *Essential Immunology* 7th Ed. Blackwell Scientific Publications, London, ISBN 0-632-02877-7 (see chapter 5 in particular).

The disclosure of the above references is incorporated herein by reference.
20 Nevertheless, an the outline of known methods is described herein.

Purification of antigens and antibodies by affinity chromatography

Antigen or antibody is bound through its free amino groups to cyanogen-
25 bromide-activated Sepharose particles. Insolubilized antibody, for example, can be used to pull the corresponding antigen out of solution in which it is present as one component of a complex mixture, by absorption to its surface. The unwanted material is washed away and the required ligand released from the affinity absorbent by disruption of the

antigen-antibody bonds by changing the pH or adding chaotropic ions such as thiocyanate. Likewise, an antigen immunosorbent can be used to absorb out an antibody from a mixture whence it can be purified by elution. The potentially damaging effect of the eluting agent can be avoided by running the anti-serum down an affinity column so prepared as to have relatively weak binding for the antibody being purified; under these circumstances, the antibody is retarded in flow rate rather than being firmly bound. If a protein mixture is separated by iso-electric focusing into discrete bands, an individual band can be used to affinity purify specific antibodies from a polyclonal antiserum.



Affinity chromatography. A column is filled with Sepharose-linked antibody. The antigen mixture is poured down the column. Only the antigen binds and is released by

change in pH for example. An antigen-linked affinity column will purify antibody obviously.

15 *Immunoassay of antigen and antibody with labelled reagents*

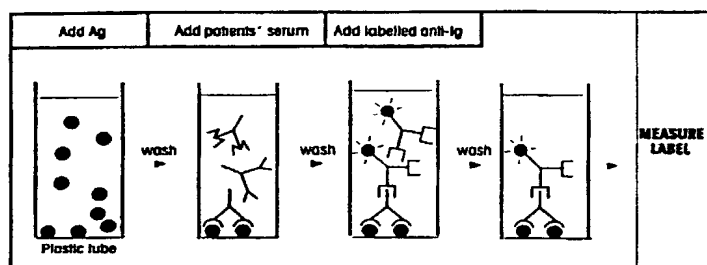
Antigen and antibody can be used for the detection of each other and a variety of immunoassay techniques have been developed in which the final read-out of the reaction involves a reagent conjugated with an appropriate label. Radiolabelling with ^{131}I , ^{125}I , is an established technique.

*Soluble Phase immunoassays**radioimmunoassay (RIA) for antigen*

- 5 The binding of radioactively labelled antigen to a limited fixed amount of antibody can be partially inhibited by addition of unlabelled antigen and the extent of this inhibition can be used as a measure of the unlabelled material added.

10 *For antibody*

- The antibody content of a serum can be assessed by the ability to bind to antigen which has been in and immobilised by physical absorption to a plastic tube or micro-agglutination tray with multiple wells; the bound
- 15 immunoglobulin may then be estimated by addition of a labelled anti-Ig raised for another species. For example, a patient's serum is added to a microwell coated with antigen, the antibodies will bind to the plastic and remaining serum proteins can be readily washed away. Bound antibody can be estimated by addition of ^{125}I -labelled purified rabbit anti IgG; after
- 20 rinsing out excess unbound reagent, the radioactivity of the tube will be a measure of the antibody content of the patient's serum. The distribution of antibody in different classes can obviously be determined by using specific antisera.

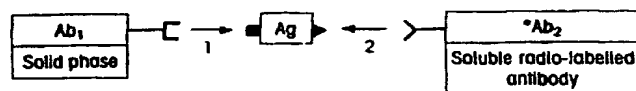


Solid phase immunoassay for antibody. By attaching antibody to the solid phase, the system can be used to assign antigen. To reduce non-specific binding of IgG to the solid phase after absorption of the first reagent, it is usual to add an irrelevant protein such as gelatin, or more recently α_1 -glycoprotein, to block any free sites on the plastic

Immunoradiometric assay for antigen

5

This differs from radioimmunoassay in the sense that the labelled reagent is used in excess. For the estimation of antigen, antibodies are coated on to a solid surface such as plastic and the test antigen solution added; after washing, the amount of antigen bound to the plastic can be estimated by adding an excess of radio-labelled antibody. The specificity of the method can be improved by the sandwich assay which uses solid phase and labelled antibodies with specificities for different parts of the antigen:



Because of health hazards and the deterioration of reagents through radiation damage, types of label other than radiosotopes have been sought.

ELISA (enzyme-linked immunosorbent assay)

Perhaps the most widespread alternative has been the use of enzymes which give a coloured reaction product, usually in solid phase assays. Enzymes such as horse radish peroxidase and phosphatase have been widely employed. A way of amplifying the phosphatase reaction is to use

NADP as a substrate to generate NAD which now acts as a coenzyme for a second enzyme system. Pyrophosphatase from *E.coli* provides a good conjugate because the enzyme is not present in tissues, is stable and gives a good reaction colour. Chemi-luminescent systems based on enzymes
5 such as luciferase can also be used.

Conjugation with the vitamin biotin is frequently used since this can readily be detected by its reaction with enzyme-linked avidin or streptavidin to which it binds with great specificity and affinity.
10

Identification of ligands by phage display

The display of proteins and polypeptides on the surface of bacteriophage (phage), fused to one of the phage coat proteins, provides a powerful tool
15 for the selection of specific ligands. This 'phage display' technique was originally used by Smith (*Science* 1985, 228, 1315-7) to create large libraries of antibodies for the purpose of selecting those with high affinity for a particular antigen. More recently, the method has been employed to present peptides, domains of proteins and intact proteins at the surface of
20 phages in order to identify ligands having desired properties.

The principles behind phage display technology are as follows:

- (i) Nucleic acid encoding the protein or polypeptide for display is cloned
25 into a phage;
- (ii) The cloned nucleic acid is expressed fused to the coat-anchoring part of one of the phage coat proteins (typically the p3 or p8 coat proteins in the case of filamentous phage), such that the foreign protein or polypeptide is displayed on the surface of the phage;

(iii) The phage displaying the protein or polypeptide with the desired properties is then selected (*e.g.* by affinity chromatography) thereby providing a genotype (linked to a phenotype) that can be sequenced, multiplied and transferred to other expression systems.

5

Alternatively, the foreign protein or polypeptide may be expressed using a phagemid vector (*i.e.* a vector comprising origins of replication derived from a phage and a plasmid) that can be packaged as a single stranded nucleic acid in a bacteriophage coat. When phagemid vectors are employed, a "helper phage" is used to supply the functions of replication and packaging of the phagemid nucleic acid. The resulting phage will express both the wild type coat protein (encoded by the helper phage) and the modified coat protein (encoded by the phagemid), whereas only the modified coat protein is expressed when a phage vector is used.

15

Methods of selecting phage expressing a protein or peptide with a desired specificity are known in the art. For example, a widely used method is "panning", in which phage stocks displaying ligands are exposed to solid phase coupled target molecules, *e.g.* using affinity chromatography.

20

Alternative methods of selecting phage of interest include SAP (Selection and Amplification of Phages; as described in WO 95/16027) and SIP (Selectively-Infective Phage; EP 614989A, WO 99/07842), which employ selection based on the amplification of phages in which the displayed ligand specifically binds to a ligand binder. In one embodiment of the SAP method, this is achieved by using non-infectious phage and connecting the ligand binder of interest to the N-terminal part of p3. Thus, if the ligand binder specifically binds to the displayed ligand, the otherwise non-infective ligand-expressing phage is provided with the parts

of p3 needed for infection. Since this interaction is reversible, selection can then be based on kinetic parameters (see Duenas *et al.*, 1996, *Mol. Immunol.* 33, 279-285).

- 5 The use of phage display to isolate ligands that bind biologically relevant molecules has been reviewed in Felici *et al.* (1995) *Biotechnol. Annual Rev.* 1, 149-183, Katz (1997) *Annual Rev. Biophys. Biomol. Struct.* 26, 27-45 and Hoogenboom *et al.* (1998) *Immunotechnology* 4(1), 1-20. Several randomised combinatorial peptide libraries have been constructed
10 to select for polypeptides that bind different targets, *e.g.* cell surface receptors or DNA (reviewed by Kay, 1995, *Perspect. Drug Discovery Des.* 2, 251-268; Kay and Paul, 1996, *Mol. Divers.* 1, 139-140). Proteins and multimeric proteins have been successfully phage-displayed as functional molecules (see EP 0349578A, EP 0527839A, EP 0589877A;
15 Chiswell and McCafferty, 1992, *Trends Biotechnol.* 10, 80-84). In addition, functional antibody fragments (*e.g.* Fab, single chain Fv [scFv]) have been expressed (McCafferty *et al.*, 1990, *Nature* 348, 552-554; Barbas *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* 88, 7978-7982; Clackson *et al.*, 1991, *Nature* 352, 624-628), and some of the shortcomings of
20 human monoclonal antibody technology have been superseded since human high affinity antibody fragments have been isolated (Marks *et al.*, 1991, *J. Mol. Biol.* 222, 581-597; Hoogenboom and Winter, 1992, *J. Mol. Biol.* 227, 381-388).

25 9. *Immunisation - Preferred protocols*

Preparation of antigen

For the preparation of monoclonal antibodies (mAbs), PrP or its peptide derivatives may be provided in an acetate buffer as described above. Antigens may be physically (by creating recombinant PrP fusion proteins) or chemically coupled to suitable carrier proteins to provide additional T cell help for immunisation in PRNP ^{+/+} mice and other rodents.

Mice of various strains, rats, hamsters or rabbits can be inoculated subcutaneously with PrP (50-100 µg/ animal), emulsified in complete/incomplete Freund's adjuvant at 3 weekly intervals (Days 0,20,41). At day 37 anti-peptide activity can be assayed by ELISA. On day 48 in the case of animals used for mAb production, a final intraperitoneal boost can be given and the animals killed for fusion 3 days later (day 50). In the case of rabbits inoculated to produce polyclonal antibodies, the animals may be bled after the final boost, and at regular subsequent intervals with or without further inoculation depending on anti-PrP titre.

10. *Monoclonal antibody preparation*

Routine methods may be used (Galfre G., and Milstein, C. 1981 *Methods in Enzymology* 73, 3-46)

Myeloma cells

The following fusion partners may be used:

5	Mouse	NSO/u	Clark M.R., and Milstein, C. 1982 <i>Somatic Cells Genetics</i> 7, 657-666
		X63/Ag 8.653	Keraney <i>et al.</i> 1979 <i>J. Immunol.</i> 123, 1548-1550
		SP2/0	Sanchez-Madrid <i>et al</i> 1983 <i>J. Immunol</i> 130, 309-312
			Bluestone 1987 <i>PNAS</i> 84, 1374
10	Rat fusions	Y3 (210.RCY3.Ag 1.2.3)YO	Galfre G., and Milstein, C. 1981 <i>Methods in Enzymology</i> 73, 3-46
	Hamster fusions	SP2/0	

Fusion procedure

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20

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Two spleens from mice that have produced high titre antibody are fused. Myeloma cells growing in exponential phase may be mixed with splenic single cell suspensions in appropriate ratios, washed free of serum, and then gently resuspended in a 50% polyethylene glycol solution at 37°C followed after 1-2 minutes with increasing volumes of serum-free medium. After a further incubation in RPMI/10% foetal calf serum (RF₁₀) at 37°C for 30 minutes, the hybridomas may be washed and resuspended in HAT medium and hybridoma growth supplements, are cultured in 200 µl flat-bottomed tissue culture wells at 37°C in 5% CO₂ enriched humidified air. The cultures remain in RF10/HAT medium for 2 weeks, and are then maintained in RF₁₀/HT medium for a further week and thereafter in RF10. At day 10-14 positive wells are screened for anti-PrP antibody by ELISA. Positive wells are then repeatedly cloned by limiting dilution until stable. Hybridomas cryopreserved in FCS 10% DMSO are stored in liquid N₂ dewars.

11. *Screening for anti-PrP antibodies in serum*

Recombinant PrP (0.5-10 μ g/well), may be dialysed against appropriate
5 coating buffer (pH 4-10) and adsorbed to standard ELISA plates for 30-60
minutes at 37°C prior to washing x4 in PBS/Tween 0.05% (PBST). After
blocking in PBS/BSA 2% with or without additional sera, dilutions of
serum are incubated in duplicate as are relevant negative and positive
controls. After washing, the peroxidase conjugated anti-IgG secondary is
10 incubated, washed and then fresh ortho-phenyl diamine (OPD) substrate
added. Finally after stopping the reaction with 3M sulphuric acid the
absorbance is measured at 492nm.

12. *Screening culture supernatants for PrP^{Sc}-specific monoclonal 15 antibodies*

This may involve a staged two day procedure. On day 1, 50 μ l of the
growing cultures may be screened for anti-PrP IgG as in the ELISA
described above. This PrP may or may not be first digested with
20 proteinase K to remove any PrP species. Positive wells in this assay may
then be screened the following day in a dot blot assay modified from *Sidle
et al* 1995. Dot blot apparatus (ELIFA, Pierce Wariner) can be used that
allows the simultaneous screening of multiple supernatants. Supernatants
can be screened for binding to recombinant β -PrP, 1% normal human
25 brain homogenate and to a pool of 1% homogenates from CJD brains
containing types 1-4, thus enabling the preferential selection of PrP^{Sc}-
specific mAbs. Thus only mAbs that bind infectious prions and not PrP^C
from normal brain will be expanded. Alternatively, culture supernatants
can be screened for preferential binding to either cellular or β -PrP, or to

synthetic peptides to which PrP^{Sc}-specific mAbs may bind. The 15B3 PrP^{Sc}-specific mAb cross-reacts with human, bovine and murine PrP^{Sc}, and its epitope has been mapped with linear synthetic peptides to three regions on the bovine PrP molecule: residues 142-148, 162-170 and 214-226 and later two of which may not be recognised by antibodies that bind to both PrP^c and PrP^{Sc} (Korth C. *et al.* 1997 *Nature* **390**, 74-77). These peptides are absorbed to ELISA plates with poly-lysine.

13. *Characterisation of mAbs*

10

Immunoglobulin subclass and culture supernatant Ig concentration can be measured by standard ELISA techniques. The fine specificity of PrP^c or PrP^{Sc} specific mAbs can be defined either by using a gridded array of overlapping human PrP peptides (synthesised commercially by Jerino Bio Tools GmbH) or by using pools of PrP synthetic peptides (synthesised individually using standard f-moc chemistry) in the standard ELISA. Measurements of the affinity of anti-PrP mAbs for their ligands can be made using surface plasmon resonance. Direct comparisons can be made of mAb binding to cellular and non-cellular-PrP molecules.

20

14. *Binding of mAbs to surface bound and intracellular PrP*

Flow cytometry and immunofluorescence microscopy may be used to study surface and intracellular PrP^c/PrP^{Sc} expression in cell lines that express surface PrP (eg EVBV lymphoblastoid, U937, K562, HEI) and peripheral blood mononuclear cells.

15. *Binding to PrP in tissue sections*

Both acetone fixed fresh frozen sections and fixed paraffin embedded sections from normal and CJD/BSE/scrapie tissue can be used to assess the usefulness of PrP binding mAbs in routine immunohistochemistry.

16. *Use of antibody in the diagnosis of a prion disease*

The detection of the disease-associated isoform of prion protein, PrP^{Sc}, in brain or other tissues from patients is thought to be diagnostic of prion disease. To distinguish PrP^{Sc} from its cellular precursor, PrP^C, requires either pre-treatment with proteinase K, which will completely digest PrP^C, but only removes a protease-sensitive N-terminal of PrP^{Sc} or, alternatively, would require an antibody which distinguished between PrP^C and PrP^{Sc}. Only one such selective antibody (Korth C. *et al.* 1997 *Nature* 390, 74-77) has yet been reported and appears to be able to selectively immunoprecipitate PrP^{Sc}. It is not clear as yet, however, whether this antibody offers any increase in diagnostic sensitivity over existing monoclonals. It is an IgM antibody and is likely to be of low affinity for PrP^{Sc}.

The PrP^C specific binding agents, especially antibodies of the invention provide an important means distinguishing PrP^{Sc} from PrP^C.

Many detection systems are available for using a monoclonal antibody to diagnose a disease. A number of possibilities are discussed below:

17. *Detection of PrP^{Sc} in body fluids or tissue homogenates*

a. Sandwich ELISA can be used to detect PrP^{Sc} in body fluids eg serum or cerebrospinal fluid (CSF). This relies on using immobilised PrP^{Sc}-specific mAbs to capture PrP^{Sc} in solution and then using biotinylated mAbs or rabbit polyclonal antiserum with specificity for alternative PrP epitopes to detect the immobilised complexes. The same techniques can be used to detect PrP^{Sc} in tissue homogenates. The PrP^C binding agents of the invention can be used in a sample pretreatment step to increase the sensitivity of such PrP^{Sc} assays. They also allow PrP^{Sc} binding agents which are not specific for PrP^{Sc} to be used in the subsequent detection step.

b. Dot blots may be used. Here tissue homogenates are placed directly on a suitable membrane and be treated with PrP^C specific binding agent of the invention to remove PrP^C. The membrane can be incubated with anti-PrP antibodies and then such binding detected using an appropriate, labelled secondary antibody. Various labelling systems, involving enzymatic, fluorescent, radioisotopic or chemiluminescent methods are commonly used.

c. Standard Western blotting techniques can be used. These methods allow not only the detection of PrP, but of specific patterns of banding following proteinase K digestion. These patterns allow the recognition of distinct strains of prions and allow, for instance, the differentiation of new variant CJD from classical CJD (see Collinge *et al.* 1996 *Nature* 383, 685-690 and international PCT patent application published as WO 98/16834).

d. Diagnostic methods may be developed based on the differential affinity of anti-PrP mAbs for PrP^C and PrP^{Sc}. Surface

to both PrP^C and PrP^{Sc} (Korth C. *et al.* 1997 *Nature* 390, 74-77). These peptides are adsorbed to ELISA plates with poly-lysine.

19. Detection of cell associated PrP^{Sc}

5

It is likely that the levels of PrP^{Sc} in peripheral blood mononuclear cells (PBMC) of vCJD patients will be low and detection will depend on optimising methods for surface and intracellular detection of PrP and then identifying lymphocyte sub-populations with the highest prion load. Anti-

10 PrP mAbs can be purified and conjugated to biotin or fluorochromes for this purpose. Dual and three colour flow cytometry can be used to identify the PrP^{Sc} bearing cell types. After surface staining by conventional techniques, intracellular PrP can be detected after fixation and permeabilisation of the cell membranes. Cellular manipulation (eg
15 stimulation of proliferation or the pharmacological blockade of intracellular secretory or endocytic pathways) may be used to enhance PrP detection. The PrP^C specific binding agents of the invention may be used to increase the sensitivity of the above methods.

20. *Immunohistochemistry*

Prion disease may be diagnosed by abnormal patterns of PrP immunoreactivity on either formalin fixed, or frozen, tissue sections using
5 established immunohistochemical detection techniques. Frozen tissue sections of whole brains (histoblots) may be treated with proteinase K or the PrP^C specific binding agents of the invention and similarly exposed to antibodies to detect patterns of PrP^{Sc} deposition which may also allow discrimination of prion strain types.

10

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CLAIMS:

1. A peptide consisting of all or a fragment or variant of an amino
5 acid sequence of a prion protein which exhibits stability when measured
by hydrogen/deuterium amide exchange at pH 5.5.
2. A peptide as claimed in Claim 1, wherein the peptide exhibits a
protection factor greater than the equilibrium constant between a native
10 state and an unfolded state; the protection factor (K_{ex}/K_{int}) being
determined by calculating amide exchange rate constants (K_{ex} from a
series 1H - ^{15}N HSQC spectra and using intrinsic amide exchange rates
(K_{int}).
- 15 3. A peptide as claimed in Claims 1 or 2, consisting of all or a
fragment or variant of an amino acid sequence from residue number 176
to 221 of one of the prion protein cellular form (PrP^c) sequences shown in
figure 5.
- 20 4. A peptide as claimed in Claim 3, consisting of all or a fragment or
variant of an amino acid sequence from residue number 179 to 218 of any
one of the said PrP^c sequences.
5. A peptide, consisting of all, or a fragment or variant of an amino
25 acid sequence of approximately ten residues which flank the disulphide
bond between Cys 179 and Cys 214 in one of the PrP^c sequences shown in
figure 5.

6. A method of making an antibody comprising administering a cellular form (PrP^c) of a prion protein or a peptide as claimed in any one of Claims 1 to 5 to an animal so as to generate an antibody response and collecting the antibody therefrom.

5

7. An antibody obtainable by a method as claimed in Claim 6; which binds preferentially to a cellular form of a prion protein rather than a non-cellular form.

10 8. An antibody which binds preferentially to a cellular form of a prion protein rather than a non-cellular form.

9. A method of making a monoclonal antibody comprising administering a cellular form (PrP^c) of a prion protein or a peptide as
15 claimed in any one of Claims 1 to 5 to an animal so as to generate an antibody response; subsequently fusing an antibody producing cell from the animal with a myeloma cell to form a hybridoma and obtaining a monoclonal antibody produced by the hybridoma.

20 10. A monoclonal antibody obtainable by a method as claimed in Claim 9 which binds preferentially to a cellular form of a prion protein rather than a non-cellular form.

11. A monoclonal antibody which binds preferentially to a cellular form
25 of a prion protein rather than a non-cellular form.

12. A method of making a binding agent capable of binding to a cellular form of a prion protein comprising exposing a peptide as claimed

in any one of Claims 1 to 5 to a sample whereby any binding agent can bind the peptide and collecting the binding agent so bound.

13. A method as claimed in Claim 12 wherein the binding agent is an
5 antibody.

14. A binding agent which binds preferentially to a cellular form of a prion protein rather than a non-cellular form.

10 15. A binding agent as claimed in Claim 14 which is obtainable by the method of Claim 12.

16. Use of an antibody or binding agent as claimed in any one of Claims 7, 10, 11, 14 or 15 in a method of detecting a cellular form of a
15 prion protein comprising exposing a sample to the antibody or binding agent and detecting binding of the antibody or binding agent to the cellular form of a prion protein.

17. Use of an antibody or binding agent as claimed in any one of
20 Claims 7, 10, 11, 14 or 15 in a method of detecting a non-cellular form of a prion protein comprising a first step of exposing a sample to the antibody or binding agent; a second step of exposing the sample to an agent which binds the non-cellular form of a prion protein; and detecting binding of the agent to the non-cellular form.

25

18. Use as claimed in Claim 17 wherein the antibody or binding agent which has bound the cellular form is separated from the sample prior to the second step.

19. Use as claimed in Claim 17 or 18 wherein the antibody or binding agent in the first and/or second step is immobilised.

20. A method of removing a cellular form of a prion protein from a sample comprising exposing the sample to an antibody or binding agent as claimed in any one of Claims 7, 10, 11, 14 or 15 and separating the sample from the antibody or binding agent which has bound the cellular form.

21. A method or use as claimed in any one of Claims 12, 13, 16 to 20 wherein the sample consists of or comprises a bodily fluid or tissue.

22. A method as claimed in Claim 21 wherein the sample is selected from one or more of blood, or a component thereof such as plasma; cerebrospinal fluid; lymph; faeces; urine; sputum; tissue from a lymph node, appendix, tonsil and spleen.

23. A method as claimed in any one of Claims 16 to 22 for use in medicine, preferably in the prevention, treatment and/or diagnosis of a prion disease.

24. A method as claimed in Claim 23 wherein the method is for use in the diagnosis of the presence of a prion disease or a predisposition to such a disease.

25. A kit comprising means for carrying out the methods of any one of Claims 16 to 24.

26. An antibody or binding agent which binds preferentially to a cellular form of a prion protein rather than a non-cellular form of a prion protein, for use in medicine, preferably in the prevention, treatment and/or diagnosis of a prion disease.

5

27. A peptide sequence of a cellular form of a prion protein which exhibits stability when measured by hydrogen/deuterium amide exchange at pH 5.5, substantially as described herein with reference to one or more of the methods and/or accompanying figures.

10

28. An antibody or binding agent which binds preferentially to a cellular form of a prion protein rather than a non-cellular form, substantially as described herein with reference to one or more of the methods and accompanying figures.

15

29. A method of using an antibody and/or binding agent as claimed in Claim 28, the method being substantially as described herein with reference to one or more of the examples and accompanying figures.

20

30. Any novel subject-matter disclosed herein.

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International Bureau



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(71) Applicant (for all designated States except US): **IMPERIAL COLLEGE INNOVATIONS LIMITED**
[GB/GB]; Sheffield Building, Imperial College, London
SW7 2AZ (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **COLLINGE, John**

[GB/GB]; MRC Prion Unit, Department of Neurogenetics, Imperial College School of Medicine at St. Mary's, Norfolk Place, London W2 1PG (GB). **CLARKE, Anthony, Russell** [GB/GB]; MRC Prion Unit, Imperial College School of Medicine at St. Mary's, Norfolk Place, London W2 1PG (GB). **WALTHO, Jonathan, Peter** [GB/GB]; 14 Rock Cottages, Cressbrook, Nr Buxton, Derbyshire SK17 8SY (GB). **JACKSON, Graham, Stuart** [GB/GB]; MRC Prion Unit, Imperial College School of Medicine at St. Mary's, Norfolk Place, London W2 1PG (GB). **HOSSZU, Laszlo, Luis, Pereira** [GB/GB]; MRC Prion Unit, Imperial College School of Medicine at St. Mary's, Norfolk Place, London W2 1PG (GB).

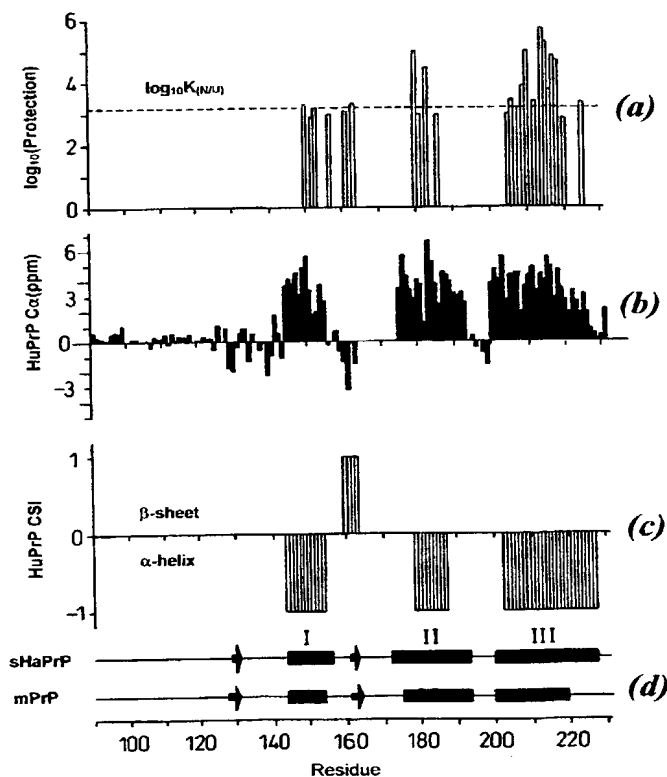
(74) Agent: **THOMAS, Philip, J., D.**; Eric Potter Clarkson, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,

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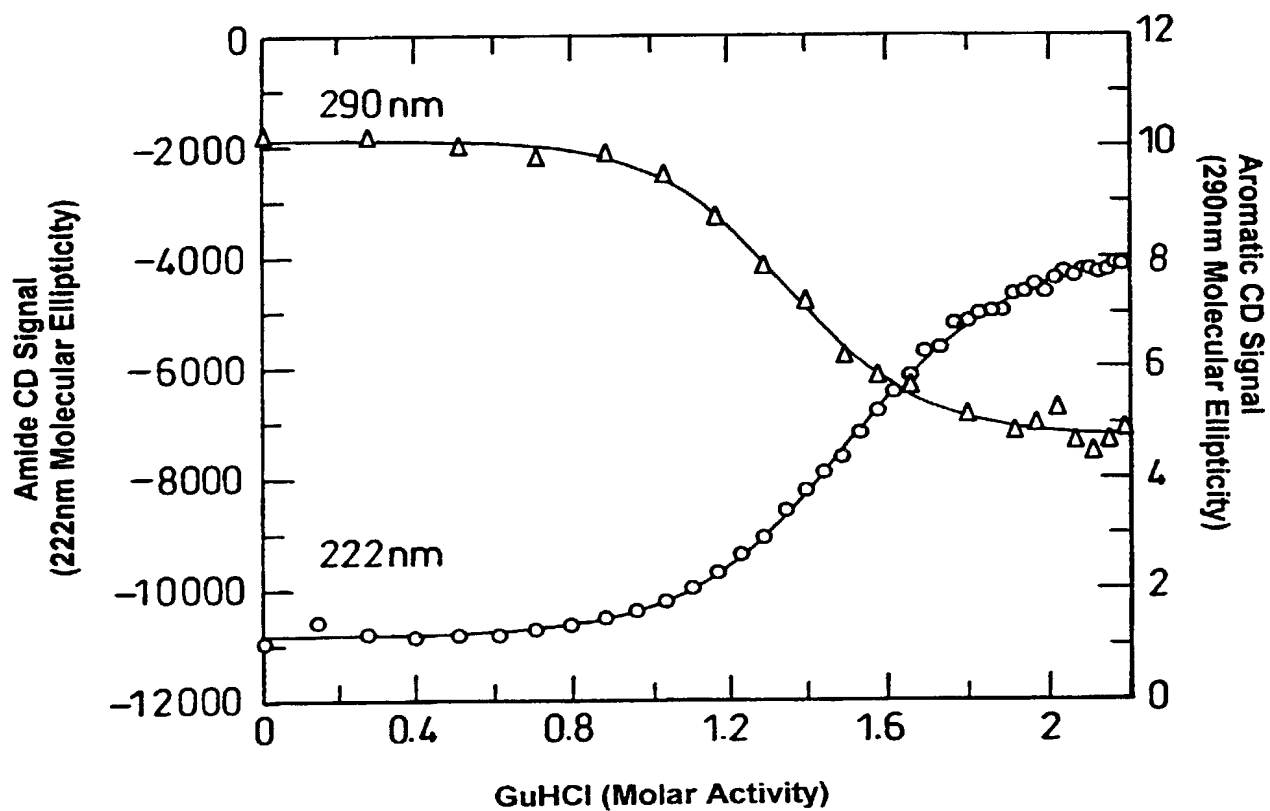
(54) Title: **BIOLOGICAL MATERIALS AND METHODS USEFUL IN THE DIAGNOSIS AND TREATMENT OF DISEASES**

(57) Abstract: The invention relates to peptides and binding agents such as antibodies and uses thereof, especially in medicine, notably in the treatment, prevention, and/or diagnosis of prion diseases.



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*Fig. 1*

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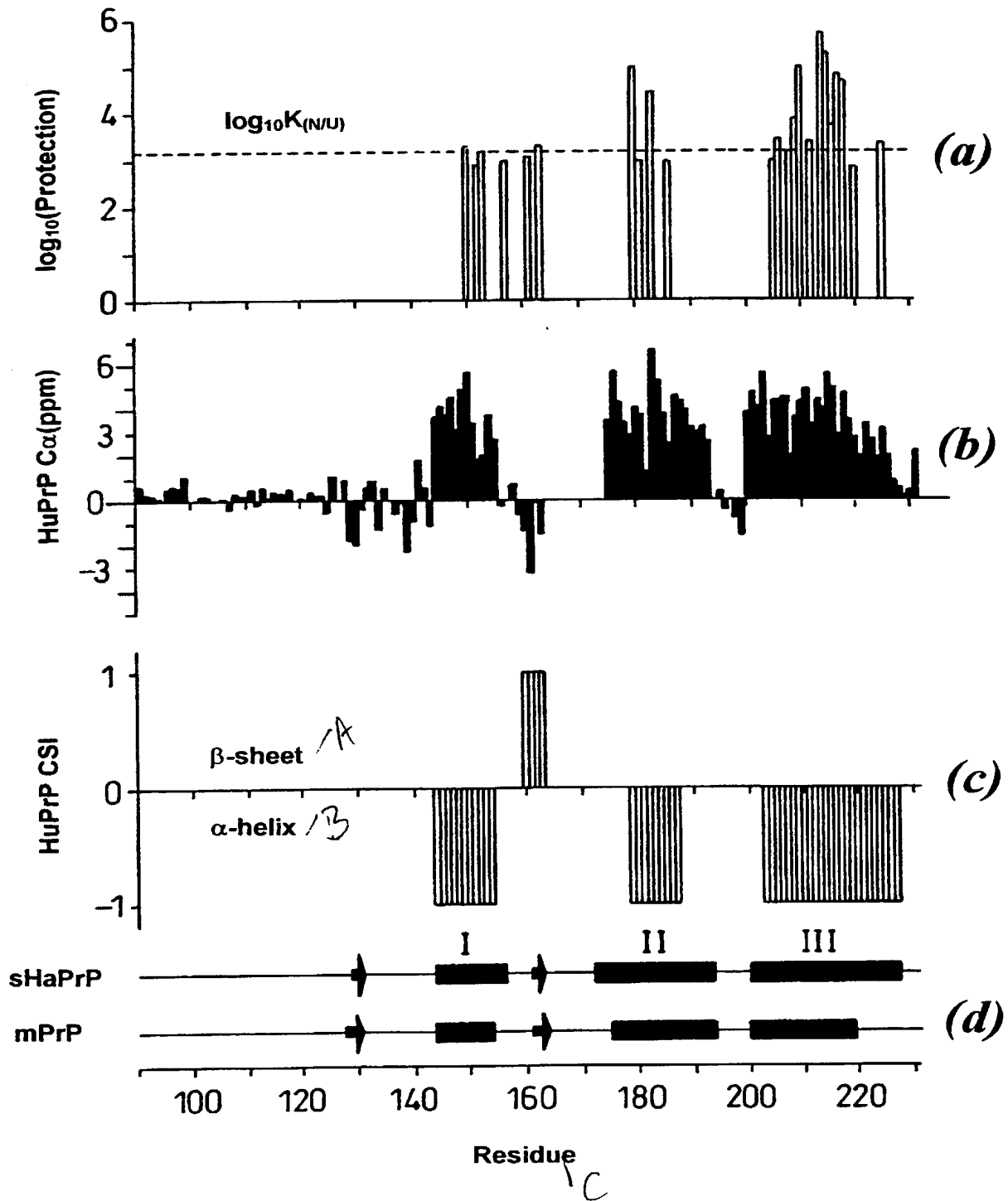
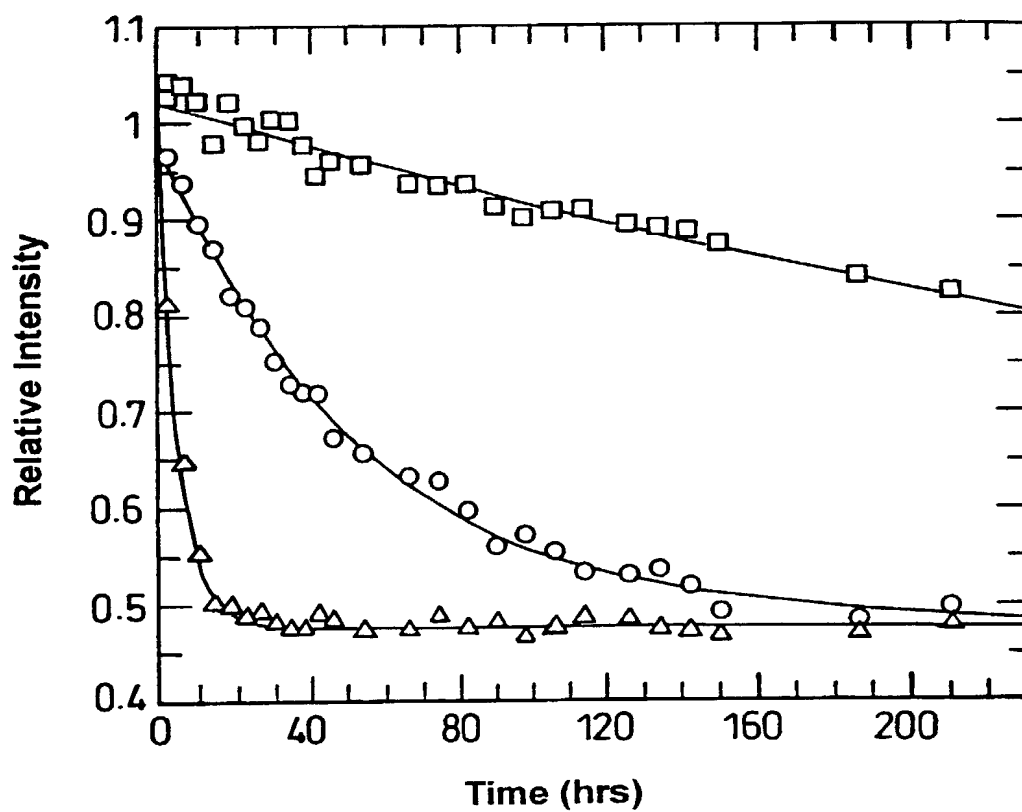


Fig. 2

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*Fig. 3*

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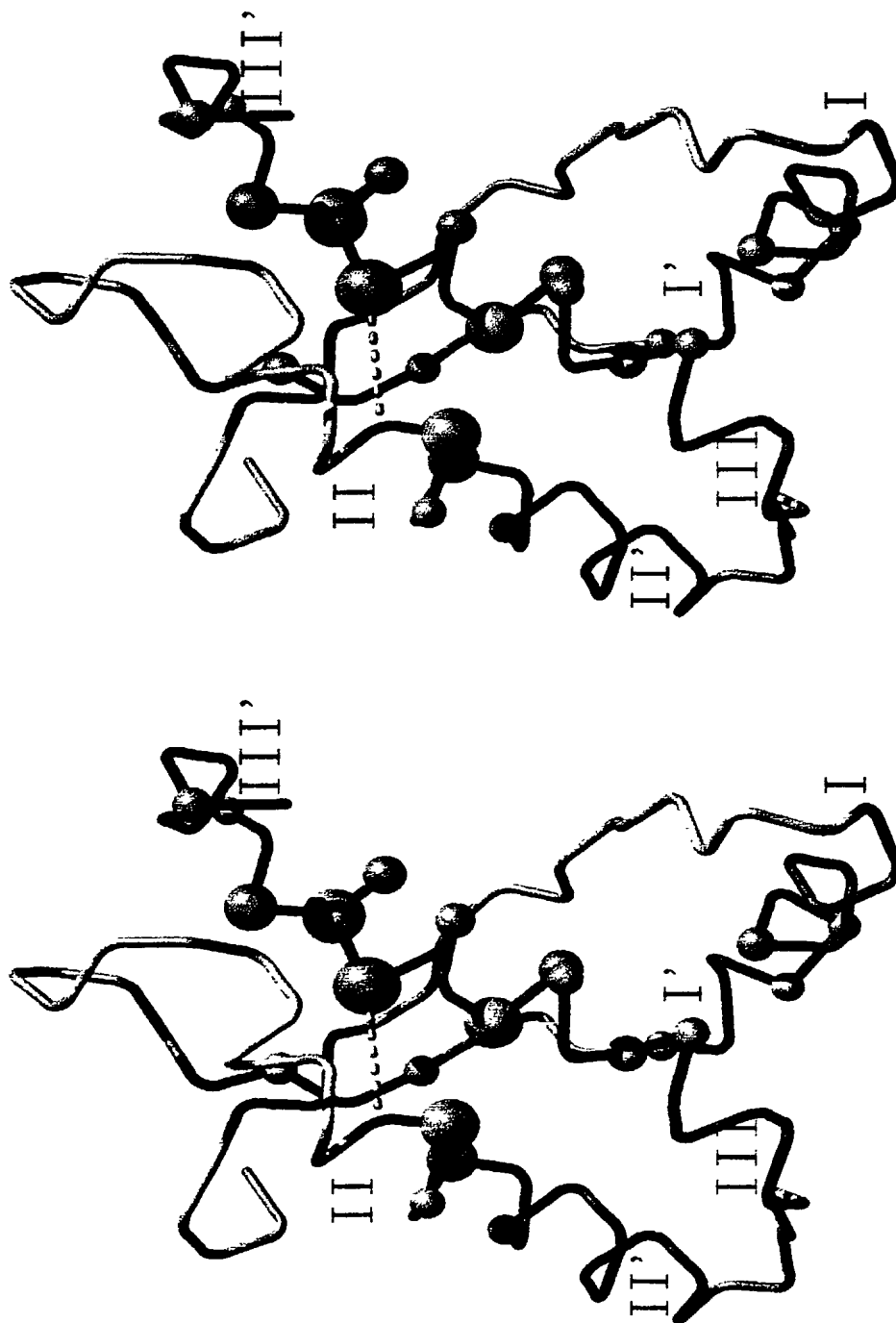


Fig. 4

	Human	-----MANLQCMQDLVATWVS DLGLCKK-RRTPGG-WNTTGGG-XYBQQ-QSFGGNRYPPQG-----GGGMQPPGGG--HQQPHGGHMQPPhGGKWQPPh-GGGHWGGGGTHSGWNTPSK-PFTNKXN	: 117
	Chimpanzee	: 117
	Orangutan N	: 117
	Gorilla	: 117
	Monkey (Gr)	V Monkey (Gr)	: 109
	Monkey (S)	- - - - - GGHWQQRH	: 124
	Rhesus Rhesus	: 117
	Gibbon	: 117
	Marmoset	F Marmoset	: 116
	Mamadyas Mamadyas	: 117
	Carpopithe	V Carpopithe	: 109
	Guereza Guereza	: 117
	Capuchin	L Capuchin	: 116
	Francoid Francoid	: 117
	Siamang Siamang	: 117
	Mouse (RMU)	I.L.A. TM.T.V. Mouse (RMU)	: 116
	Mouse (Sh)	I.L.A. TM.T.V. Mouse (Sh)	: 116
	Mouse (Lg)	I.L.A. TM.T.V. Mouse (Lg)	: 116
	Banster (C)	SI.L.A. T.V. Banster (C)	: 117
	Cow	MVKSHI.S.I. M.V. ... Cow	: 126
	Sheep	---MVKSHI.S.I. M.V. ... Sheep	: 114
	Antelope	MVKSHI.S.I. M.V. ... Antelope	: 118
	Kudu	MVKSHI.S.I. M.VA. ... Kudu	: 126
	Goat	MVKSHI.S.I. M.V. ... Goat	: 118
	Pig	MVKSHI.G.I. A.I. ... Pig	: 119
	Polecat	MVKSHI.S.L. I.F. ... Polecat	: 119
	Dog	MVKSHI.G.I. V. ... Dog	: 118
	Rabbit	H.Y.L. V. ... Rabbit	: 116
	Maraspial	MGRIQ.X.I.IV. ... Maraspial	: 120
	Chicken	MPAMARILTT.CLA.LACT.VA.S.OKG.S.GGWGA.H.O.SPEPO. ---ENPGFHTHPGTPTPHZ.Y.NP.YPTHNPQTPTPH.Z.NP.YTGWQJ.INSS.SY.N.---W.P.J.	: 128

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E221

V176

Human	MACAAAAGAVVGLGOTLGSMSRPIHFGSDIEDAYIRENHERIFNOVITRPMDEISNQNFVDCNITIKSTV-TTITKENTTETDVRGZRVVZOMCITOIERES--QAIY--QKSSMWLFSSPP	245
ChimpanzeeQ..S.....	245
OrangutanY.....V.Q.....	245
GorillaQ.....	245
Monkey (Gr)	L..N..N..Y.....V.Q.....K..	237
Monkey (S)	L..N..N..Y..S..V.Q.....K..	252
Rhesus	L..N..N..Y.....V.Q.....K..	245
GibbonQ..S.....	245
Macaque (S)	L..N..N..Y.....V.Q.....K..	245
Macaque (C)	L..N..N..Y.....V.Q.....K..	245
Macaque (J)	L..N..N..Y.....V.Q.....K..	245
Macaque (P)	L..N..N..Y.....V.Q.....K..	245
Marmoset	V.....V.Q.N.....K..	244
Hamadryas	L..N..N..Y.....V.Q.....K..	245
Cercopithe	L..N..N..Y.....V.Q.....K..	237
Guereza	L..N..N..Y.....V.Q.....K..	245
Capuchin	L..N..N..Y.....V.Q.....	244
Francoisi	L..N..N..Y.....V.Q.....K..	245
SiamangQ..S.....	245
Mouse (RML)	V..M..N.W..Y.....V.Q.....V.QK..DGR.S..T..	246
Mouse (Sh)	M..N.W..Y.....V.Q.....V.QK..DGR.S..T..	246
Mouse (Lg)	M..N.W..Y.....V.Q.....V.QK..DGR.S..T..	246
Hamster (C)	ML..N.W..N..V.Q.N.....V.QK..DGR.S.A..	246
Cow	L..N..N..Y.....V.Q.....I..I..A.VI..	254
Sheep	L..N..N..Y.....V.Q.....I..I..A.VI..	246
Antelope	N..L..N..Y.....V.Q.N..T.....V.QK..A.VI..	246
Kudu	L..N..N..Y.....V.Q.....I..I..A.VI..	254
Goat	L..N..N..Y.....V.Q.....I..I..A.VI..	246
Pig	L..N..N..Y.....V.Q.....I..I..A.VI..	247
Polecat	L..N..N..Y.....V.Q.....I..I..A.VI..	246
Dog	L..N..N..Y.....V.Q.....I..I..A.VI..	246
Rabbit	L..N..N..Y.....V.Q.....I..I..A.VI..	244
Marsupial	V..NE..QI..M..I.Q..S.....V..T..QA..I..E..AA..AIN..AF..A..	248
Chicken	AM..RV..GAY..D..PD..Y..HWS..SA..R..DISSEVP..DV..A..F..VTEISIGPAK..NTSEVAANAQT..VEM..NKVV..KVI..MCTV..Q..REYRLA..GIQ..HPADT..	261

Fig. 5 (Part 2 of 3)

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Human	:	VILL-ISFLIFLIVG----	:	260
Chimpanzee	:*---	:	260
Orangutan	:*---	:	260
Gorilla	:*---	:	260
Monkey (Gr)	:*---	:	252
Monkey (S)	:*---	:	267
Rhesus	:*---	:	260
Gibbon	:*---	:	260
Macaque (S)	:*---	:	260
Macaque (C)	:*---	:	260
Macaque (J)	:*---	:	260
Macaque (P)	:*---	:	260
Marmoset	:*---	:	259
Hamadryas	:*---	:	260
Cercopithe	:*---	:	252
Guereza	:*---	:	260
Capuchin	:*---	:	259
Francoisi	:*---	:	260
Siamang	:*---	:	260
Mouse (RML)	:*---	:	261
Mouse (Sh)	:*---	:	261
Mouse (Lg)	:*---	:	261
Hamster (C)	:*---	:	261
Cow	:*---	:	269
Sheep	:*---	:	256
Antelope	:*---	:	261
Kudu	:*---	:	269
Goat	:*---	:	261
Pig	:L.....*---	:	262
Polecat	:L..L.....*---	:	262
Dog	:L..L.....---	:	261
Rabbit	:*---	:	259
Marsupial	:	.T..FL.....S.---	:	264
Chicken	:	WLAVLLLL.TT.FAMH---	:	277

Fig. 5 (Part 3 of 3)

ATTORNEY FILE NO. 20020011.ORI

SUPPLEMENTAL DECLARATION, POWER OF ATTORNEY, AND PETITION

As below named inventors, we hereby declare that: our residences, post office addresses and citizenships are as stated below next to our names; that we verily believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled:

FRAGMENTS OF CELLULAR PRION PROTEIN AND METHODS
USEFUL IN THE DIAGNOSIS AND TREATMENT OF PRION DISEASES

Serial No. 10/031,975, filed January 24, 2002.

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to in the Oath or Declaration.

We acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, Section 1.56.

We hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign Application(s):

International Application No. PCT/GB00/02873

International Filing Date: 25 July 2000

Priority Date Claimed: 27 July 1999

Entitled: FRAGMENTS OF CELLULAR PRION PROTEIN AND METHODS
USEFUL IN THE DIAGNOSIS AND TREATMENT OF PRION
DISEASES

We hereby appoint NIKOLAI & MERSEREAU, P.A., (Customer Number 23595), a professional association, consisting of the following attorneys/agents and the following attorneys/agents individually: Thomas J. Nikolai, Registration No. 19,283; Charles G. Mersereau, Registration No. 26,205; and Steven E. Kahm, Registration No. 30,860 of 820 International Centre, 900 Second Avenue South, Minneapolis, Minnesota 55402-3813; Telephone No. (612) 339-7461, our attorneys/agents with full power of substitution and revocation to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Please direct all phone calls and correspondence to: C. G. Mersereau, Esq. at NIKOLAI & MERSEREAU, P.A., 820 International Centre, 900 Second Avenue South, Minneapolis, Minnesota 55402-3813, Telephone: (612) 339-7461.

Post Office Address:
MRC Prion Unit
Imperial College School of Medicine
St. Mary's Campus
Norfolk Place
London
W2 1PG
United Kingdom

Name: 50 L. Laszlo
Laszlo Luis Periera Hosszu

Date: 24.06.02

Residence:
London, United Kingdom GBN

Citizenship:
British

Post Office Address:
MRC Prion Unit
Imperial College School of Medicine
St. Mary's Campus
Norfolk Place
London
W2 1PG
United Kingdom

10/03/97

10/031975

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SEQUENCE LISTING

<110> Imperial College Innovations Limited
<110> John Collinge
<110> Anthony Russell Clarke
<110> Jonathan Peter Waltho
<110> Graham Stuart Jackson
<110> Laszlo Luis Pereira Hosszu
<120> Biological Materials And Methods Useful In The Diagnosis And
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20

25

30

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40

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Gln Gly Gly Gly Thr His Ser Gln Trp Asn Lys Pro Ser Lys Pro Lys

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Pro His Gly Gly Gly Trp Gly Gln Gly Gly Gly Thr His Asn Gln Trp

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Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly

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His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp

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Asn Gln Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln

180 185 190

His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp

195 200 205

Val Lys Met Met Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr

210 215 220

Glu Lys Glu Ser Gln Ala Tyr Tyr Gln Arg Gly Ser Ser Met Val Leu

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Phe Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu

245 250 255

Ile Val Gly His

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<211> 260

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<213> Macaca fuscata

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Leu Phe Val Ala Thr Trp Ser Asp Leu Gly Leu Cys Lys Lys Arg Pro

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Lys Pro Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly

35

40

45

Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly

50

55

60

Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly

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70

75

80

Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly

85

90

95

Gln Gly Gly Gly Thr His Asn Gln Trp His Lys Pro Ser Lys Pro Lys

100

105

110

Thr Ser Met Lys His Met Ala Gly Ala Ala Ala Ala Gly Ala Val Val

115

120

125

Gly Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Leu

130

135

140

Ile His Phe Gly Asn Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met

145

150

155

160

Tyr Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser

165

170

175

Asn Gln Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln

180

185

190

His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp

195

200

205

Val Lys Met Met Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr

210

215

220

Glu Lys Glu Ser Gln Ala Tyr Tyr Gln Arg Gly Ser Ser Met Val Leu

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Phe Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu

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Ile Val Gly His

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25

30

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

Lys Pro Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly

35

40

45

Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly

50

55

60

Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly

65

70

75

80

Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly

85

90

95

Gln Gly Gly Gly Thr His Asn Gln Trp His Lys Pro Ser Lys Pro Lys

100

105

110

Thr Ser Met Lys His Met Ala Gly Ala Ala Ala Ala Gly Ala Val Val

115

120

125

Gly Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Leu

130

135

140

Ile His Phe Gly Asn Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met

145

150

155

160

Tyr Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser

165

170

175

Asn Gln Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln

180

185

190

Callithrix jacchus

His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp

195

200

205

Val Lys Met Met Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr

210

215

220

Glu Lys Glu Ser Gln Ala Tyr Tyr Gln Arg Gly Ser Ser Met Val Leu

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Phe Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu

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Ile Val Gly His

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Leu Phe Val Ala Thr Trp Ser Asp Leu Gly Leu Cys Lys Lys Arg Pro

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30

Lys Pro Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly

35

40

45

205

Glu Lys Glu Ser Gln Ala Tyr Tyr Gln Arg Gly Ser Ser Met Val Leu

225 230 235 240

Phe Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu

245 250 255

Ile Val Gly His

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<210> 15

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<212> PRT

<213> Cercopithecus patas

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1 5 10 15

Val Phe Val Ala Thr Trp Ser Asp Leu Gly Leu Cys Lys Lys Arg Pro

20 25 30

Lys Pro Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly

35 40 45

Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly

50 55 60

Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly

65 70 75 80

11

[illegible]

95

Abstract

110

11/10/2000 12:00 PM
From: Aude@worldnet.att.net

125

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140

160

1. **Introduction**

175

190

205

220

240

Thr Ser Met Lys His Met Ala Gly Ala Ala Ala Ala Gly Ala Val Val

115

120

125

Gly Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Leu

130

135

140

Ile His Phe Gly Asn Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met

145

150

155

160

Tyr Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser

165

170

175

Asn Gln Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln

180

185

190

His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp

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200

205

Val Lys Met Met Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr

210

215

220

Glu Lys Glu Ser Gln Ala Tyr Tyr Gln Arg Gly Ser Ser Met Val Leu

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Phe Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu

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Ile Val Gly His

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<211> 259

<212> PRT

<213> Cebus apella

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Leu Phe Val Ala Thr Trp Ser Asp Leu Gly Leu Cys Lys Lys Arg Pro

20 25 30

Lys Pro Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly

35 40 45

Ser Pro Gly Gly Asn Leu Tyr Pro Pro Gln Gly Gly Gly Trp Gly Gln

50 55 60

Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln

65 70 75 80

Pro His Gly Gly Ser Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln

85 90 95

Gly Gly Gly Thr His Asn Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr

100 105 110

Ser Met Lys His Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly

115 120 125

.....

Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Leu Ile

130 135 140

His Phe Gly Asn Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr

145 150 155 160

Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn

165 170 175

Gln Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln His

180 185 190

Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Val

195 200 205

Lys Met Met Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr Glu

210 215 220

Arg Glu Ser Gln Ala Tyr Tyr Gln Arg Gly Ser Ser Met Val Leu Phe

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Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu Ile

245 250 255

Val Gly His

<210> 18

<211> 260

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..

Ile His Phe Gly Asn Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met

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Tyr Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser

165 170 175

Asn Gln Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln

180 185 190

His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp

195 200 205

Val Lys Met Met Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr

210 215 220

Glu Lys Glu Ser Gln Ala Tyr Tyr Gln Arg Gly Ser Ser Met Val Phe

225 230 235 240

Phe Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu

245 250 255

Ile Val Gly His

260

<210> 19

<211> 260

<212> PRT

<213> Hylobates syndactylus

<400> 19

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Met Pro Ala Ala Met Ala Met Ala Asn Leu Gly Cys Trp Met Leu Val

1	5	10	15
---	---	----	----

Leu Phe Val Ala Thr Trp Ser Asp Leu Gly Leu Cys Lys Lys Arg Pro

20 25 30

Lys Pro Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly

35 40 45

Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly

50 55 60

Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly

65 70 75 80

Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly

85 90 95

Gln Gly Gly Gly Thr His Ser Gln Trp Asn Lys Pro Ser Lys Pro Lys

100 105 110

Thr Asn Met Lys His Met Ala Gly Ala Ala Ala Ala Gly Ala Val Val

115 120 125

Gly Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Ile

130 135 140

Ile His Phe Gly Ser Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met

145 150 155 160

His Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Met Asp Gln Tyr Ser

165

170

175

Ser Gln Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln

180

185

190

His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp

195

200

205

Val Lys Met Met Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr

210

215

220

Glu Arg Glu Ser Gln Ala Tyr Tyr Gln Arg Gly Ser Ser Met Val Leu

225

230

235

240

Phe Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu

245

250

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Ile Val Gly His

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<212> PRT

<213> Mus musculus

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Leu Phe Val Thr Met Trp Thr Asp Val Gly Leu Cys Lys Lys Arg Pro

20

25

30

Lys Pro Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly

35

40

45

Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln Gly Gly Thr Trp Gly Gln

50

55

60

Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Ser Trp Gly Gln

65

70

75

80

Pro His Gly Gly Ser Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln

85

90

95

Gly Gly Gly Thr His Asn Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr

100

105

110

Asn Leu Lys His Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly

115

120

125

Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Val Ser Arg Pro Met Ile

130

135

140

His Phe Gly Asn Asp Trp Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr

145

150

155

160

Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn

165

170

175

Lys Pro Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly
 35 40 45

Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln Gly Gly Thr Trp Gly Gln
 50 55 60

Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Ser Trp Gly Gln
 65 70 75 80

Pro His Gly Gly Ser Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln
 85 90 95

Gly Gly Gly Thr His Asn Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr
 100 105 110

Asn Leu Lys His Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly
 115 120 125

Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Met Ile
 130 135 140

His Phe Gly Asn Asp Trp Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr
 145 150 155 160

Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn
 165 170 175

Gln Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln His
 180 185 190

Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln Gly Gly Thr Trp Gly Gln
50 55 60

Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Ser Trp Gly Gln
65 70 75 80

Pro His Gly Gly Ser Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln
85 90 95

Gly Gly Gly Thr His Asn Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr
100 105 110

Asn Phe Lys His Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly
115 120 125

Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Met Ile
130 135 140

His Phe Gly Asn Asp Trp Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr
145 150 155 160

Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn
165 170 175

Gln Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln His
180 185 190

Thr Val Val Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Val
195 200 205

Gln Lys Glu Ser Gln Ala Tyr Tyr Asp Gly Arg Arg Ser Ser Ala Val
225 230 235 240

Leu Phe Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe
245 250 255

Leu Ile Val Gly His
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<210> 24
<211> 269
<212> PRT
<213> Bos taurus

<400> 24
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1 5 10 15

Leu Phe Val Ala Met Trp Ser Asp Val Gly Leu Cys Lys Lys Arg Pro
20 25 30

Lys Pro Gly Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln
35 40 45

Gly Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln Gly Gly Gly Trp
50 55 60

Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp
65 70 75 80

$$\frac{1}{\Gamma(\alpha)} \int_0^t (t-\tau)^{\alpha-1} f(\tau) d\tau = I^\alpha f(t), \quad t \in [0, T],$$

11

1. Introduction

11

Leu Gly Ser Ala Met Ser Arg Pro Leu Ile His Phe Gly Ser Asp Tyr
145 150 155 160

Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr Arg Tyr Pro Asn Gln Val
165 170 175

Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn Gln Asn Asn Phe Val His
180 185 190

Asp Cys Val Asn Ile Thr Val Lys Gln His Thr Val Thr Thr Thr Thr
195 200 205

Lys Gly Glu Asn Phe Thr Glu Thr Asp Ile Lys Met Met Glu Arg Val
210 215 220

Val Glu Gln Met Cys Ile Thr Gln Tyr Gln Arg Glu Ser Glu Ala Tyr
225 230 235 240

Tyr Gln Arg Gly Ala Ser Val Ile Leu Phe Ser Ser Pro Pro Val Ile
245 250 255

Leu Leu Ile Ser Phe Leu Ile Phe Leu Ile Val Gly His
260 265

<210> 28

<211> 261

<212> PRT

<213> Capra hircus

<400> 28

Met Pro Ala Ala Met Val Lys Ser His Ile Gly Ser Trp Ile Leu Val

1 5 10 15

Leu Phe Val Ala Met Trp Ser Asp Val Gly Leu Cys Lys Lys Arg Pro

20 25 30

Lys Pro Gly Gly Gly Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln

35 40 45

Gly Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln Gly Gly Gly Gly Trp

50 55 60

Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp

65 70 75 80

Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Gly

85 90 95

Trp Gly Gln Gly Gly Ser His Ser Gln Trp Asn Lys Pro Ser Lys Pro

100 105 110

Lys Thr Asn Met Lys His Val Ala Gly Ala Ala Ala Ala Gly Ala Val

115 120 125

Val Gly Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro

130 135 140

Leu Ile His Phe Gly Asn Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn

145 150 155 160

.....

Met Tyr Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr
165 170 175

Ser Asn Gln Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Val Lys
180 185 190

Gln His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr
195 200 205

Asp Ile Lys Ile Met Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln
210 215 220

Tyr Gln Arg Glu Ser Gln Ala Tyr Tyr Gln Arg Gly Ala Ser Val Ile
225 230 235 240

Leu Phe Ser Pro Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe
245 250 255

Leu Ile Val Gly His
260

<210> 29

<211> 262

<212> PRT

<213> Sus scrofa

<400> 29

Met Pro Ala Ala Met Val Lys Ser His Ile Gly Gly Trp Ile Leu Val
1 5 10 15

Tyr Ser Asn Gln Asn Ser Phe Val His Asp Cys Val Asn Ile Thr Val
 180 185 190

Lys Gln His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu
 195 200 205

Thr Asp Val Lys Met Ile Glu Arg Val Val Glu Gln Met Cys Ile Thr
 210 215 220

Gln Tyr Gln Lys Glu Tyr Glu Ala Tyr Ala Gln Arg Gly Ala Ser Val
 225 230 235 240

Ile Leu Phe Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Leu
 245 250 255

Phe Leu Ile Val Gly His
 260

<210> 30
 <211> 262
 <212> PRT
 <213> Mustela putorius

<400> 30
 Met Pro Ala Ala Met Val Lys Ser His Ile Gly Ser Trp Leu Leu Val
 1 5 10 15

Leu Phe Val Ala Thr Trp Ser Asp Ile Gly Phe Cys Lys Lys Arg Pro
 20 25 30

190

of 1.25 Watts per inch

Abstract

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Gln His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr
195 200 205

Asp Met Lys Ile Met Glu Arg Val Val Glu Gln Met Cys Val Thr Gln
210 215 220

Leu Phe Ser Pro Pro Pro Val Ile Leu Leu Ile Ser Leu Leu Ile Leu

245 250 255

<210>	32
<211>	259
<212>	PRT
<213>	Oryctolagus cuniculus

Leu Phe Val Ala Thr Trp Ser Asp Val Gly Leu Cys Lys Lys Arg Pro
20 25 30

Ser Ser Pro Gly Gly Asn Arg Tyr Pro Pro Gln Gly Gly Gly Trp Gly
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Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly
65 70 75 80

Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly
85 90 95

Gln Gly Gly Thr His Asn Gln Trp Gly Lys Pro Ser Lys Pro Lys Thr
100 105 110

Ser Met Lys His Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly
115 120 125

Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Leu Ile
130 135 140

His Phe Gly Asn Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr
145 150 155 160

Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn
165 170 175

Gln Asn Ser Phe Val His Asp Cys Val Asn Ile Thr Val Lys Gln His
180 185 190

Thr Val Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Ile
195 200 205

Lys Ile Met Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr Gln
210 215 220

$$6\text{H}^+ + \frac{1}{2}\text{O}_2 + \frac{1}{2}\text{S}_2\text{O}_8^{2-} \rightarrow 2\text{H}_2\text{SO}_4$$

225 230 235 240

Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu Ile

245 250 255

Val Gly His

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<212> PRT

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Leu Phe Ile Val Thr Trp Ser Asp Leu Gly Leu Cys Lys Lys Pro Lys

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Pro Arg Pro Gly Gly Gly Trp Asn Ser Gly Gly Ser Asn Arg Tyr Pro

35 40 45

Gly Gln Pro Gly Ser Pro Gly Gly Asn Arg Tyr Pro Gly Trp Gly His

50 55 60

Pro Gln Gly Gly Gly Thr Asn Trp Gly Gln Pro His Pro Gly Gly Ser

65 70 75 80

Asn Trp Gly Gln Pro His Pro Gly Gly Ser Ser Trp Gly Gln Pro His
85 90 95

Gly Gly Ser Asn Trp Gly Gln Gly Gly Tyr Asn Lys Trp Lys Pro Asp
100 105 110

Lys Pro Lys Thr Asn Leu Lys His Val Ala Gly Ala Ala Ala Ala Gly
115 120 125

Ala Val Val Gly Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser
130 135 140

Arg Pro Val Ile His Phe Gly Asn Glu Tyr Glu Asp Arg Tyr Tyr Arg
145 150 155 160

Glu Asn Gln Tyr Arg Tyr Pro Asn Gln Val Met Tyr Arg Pro Ile Asp
165 170 175

Gln Tyr Ser Ser Gln Asn Asn Phe Val His Asp Cys Val Asn Ile Thr
180 185 190

Val Lys Gln His Thr Thr Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr
195 200 205

Glu Thr Asp Ile Lys Ile Met Glu Arg Val Val Glu Gln Met Cys Ile
210 215 220

Thr Gln Tyr Gln Ala Glu Tyr Glu Ala Ala Ala Gln Arg Ala Tyr Asn
225 230 235 240

• • •

Gly Tyr Pro Gly Trp Gly Gln Gly Tyr Asn Pro Ser Ser Gly Gly Ser
100 105 110

Tyr His Asn Gln Lys Pro Trp Lys Pro Pro Lys Thr Asn Phe Lys His
115 120 125

Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly Leu Gly Gly
130 135 140

Tyr Ala Met Gly Arg Val Met Ser Gly Met Asn Tyr His Phe Asp Ser
145 150 155 160

Pro Asp Glu Tyr Arg Trp Trp Ser Glu Asn Ser Ala Arg Tyr Pro Asn
165 170 175

Arg Val Tyr Tyr Arg Asp Tyr Ser Ser Pro Val Pro Gln Asp Val Phe
180 185 190

Val Ala Asp Cys Phe Asn Ile Thr Val Thr Glu Tyr Ser Ile Gly Pro
195 200 205

Ala Ala Lys Lys Asn Thr Ser Glu Ala Val Ala Ala Ala Asn Gln Thr
210 215 220

Glu Val Glu Met Glu Asn Lys Val Val Thr Lys Val Ile Arg Glu Met
225 230 235 240

Cys Val Gln Gln Tyr Arg Glu Tyr Arg Leu Ala Ser Gly Ile Gln Leu
245 250 255

